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RESEARCH PAPER

Genetic and molecular characterization of three novel *S*-haplotypes in sour cherry (*Prunus cerasus* L.)

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Abstract

Tetraploid sour cherry (*Prunus cerasus* L.) exhibits gametophytic self-incompatibility (GSI) whereby the specificity of self-pollen rejection is controlled by alleles of the stylar and pollen specificity genes, *S-RNase* and *SFB* (*S* haplotype-specific F-box protein gene), respectively. As sour cherry selections can be either self-compatible (SC) or self-incompatible (SI), polyploidy *per se* does not result in SC. Instead the genotype-dependent loss of SI in sour cherry is due to the accumulation of non-functional *S*-haplotypes. The presence of two or more non-functional *S*-haplotypes within sour cherry 2*x* pollen renders that pollen SC. Two new *S*-haplotypes from sour cherry, S_{33} and S_{34}, that are presumed to be contributed by the *P. fruticosa* species parent, the complete *S-RNase* and *SFB* sequences of a third *S*-haplotype, S_{35}, plus the presence of two previously identified sweet cherry *S*-haplotypes, S_{14} and S_{16} are described here. Genetic segregation data demonstrated that the S_{16}-, S_{33}-, S_{34}-, and S_{35}-haplotypes present in sour cherry are fully functional. This result is consistent with our previous finding that 'hetero-allelic' pollen is incompatible in sour cherry. Phylogenetic analyses of the *SFB* and *S-RNase* sequences from available *Prunus* species reveal that the relationships among *S*-haplotypes show no correspondence to known organismal relationships at any taxonomic level within *Prunus*, indicating that polymorphisms at the *S*-locus have been maintained throughout the evolution of the genus. Furthermore, the phylogenetic relationships among *SFB* sequences are generally incongruent with those among *S-RNase* sequences for the same *S*-haplotypes. Hypotheses compatible with these results are discussed.

Key words: *Prunus cerasus*, self-incompatibility, *SFB*, *S-RNase*.

Introduction

Gametophytic self-incompatibility (GSI) is a common genetic mechanism that promotes outcrossing in flowering plants (de Nettancourt, 2001). In GSI, self-incompatibility (SI) is determined by a single multi-allelic locus, called the *S*-locus, which contains a minimum of two genes, one (stylar-*S*) controlling stylar specificity and the other (pollen-*S*) controlling pollen specificity of the SI reaction. The stylar-*S* gene in three plant families, the Solanaceae, Plantaginaceae, and Rosaceae encodes a ribonuclease (S-RNase; Anderson *et al*., 1986; McClure *et al*., 1989; Sassa *et al*., 1992; Xue *et al*., 1996), which is expressed in the pistil and specifically degrades the RNA of incompatible pollen (McClure *et al*., 1990). The pollen-*S* gene encodes an F-box protein named *S*-locus F-box protein (SLF) in *Antirrhinum* (Lai *et al*., 2002), *Petunia inflata* (Sijacic *et al*., 2004), and *Prunus mume* (Entani *et al*., 2003), or *S* haplotype-specific F-box protein (SFB) in *Prunus dulcis*, *P. mume*, *P. avium*, *P. spinosa*, and *P. cerasus* (Ushijima *et al*., 2003; Yamane *et al*., 2003*b*; Ikeda *et al*., 2004*a;* Nunes *et al*., 2006). Despite having similar or even identical names in Solanaceae and *Prunus* the pollen gene is not orthologous (Wheeler and Newbigin, 2007).

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Abbreviations: GSI, gametophytic self-incompatibility; SI, self-incompatible; SC, self-compatible; SFB, *S* haplotype-specific F-box protein.

Within *Prunus* (Rosaceae), cherry represents a natural diploid–tetraploid series with the tetraploid sour cherry (*P. cerasus*) arising through hybridization between sweet cherry (*P. avium*) and the tetraploid ground cherry (*P. fruticosa*) (Olden and Nybom, 1968). Like sweet cherry, sour cherry exhibits an S-RNase-based GSI system (Yamane *et al*., 2001; Hauck *et al*., 2002; Tobutt *et al*., 2004), however, in contrast to sweet cherry, natural sour cherry selections include both SI and self-compatible (SC) types (Redalen, 1984; Lansari and Iezzoni, 1990). This genotype-dependent loss of SI in sour cherry indicates that genetic changes, and not polyploidy *per se*, cause the breakdown of SI. The genetic switch from SI to SC in sour cherry results from the accumulation of non-functional *S*-haplotypes according to the 'one-allele-match model' (Hauck *et al*., 2006*b*). In this model, the match between a functional pollen-S in the 2*x* pollen and its cognate functional S-RNase in the style, results in an incompatible reaction. A similar reaction would occur regardless of whether the pollen contained a single functional pollen-*S* gene or two different pollen-*S* genes. The absence of any functional match results in a compatible reaction. Thus for successful self-fertilization, the 2*x* pollen must contain two non-functional *S*-haplotypes. Recently, Huang *et al*. (2008) reported competitive interaction in a SC selection of tetraploid *Prunus pseudocerasus*, raising the possibility that the SC mechanism between these two tetraploid *Prunus* species could be different. However, although the data in Huang *et al*. (2008) is consistent with hetero-allelic pollen being SC, homo-allelic pollen (e.g. S_1S_1, S_5S_5, or S_7S_7) was not shown to be successful in a compatible cross and unsuccessful in an incompatible cross. Therefore, it is possible that the SC in *P. pseudocerasus* could be caused by mutations in other genes critical for the SI reaction.

Six *S*-haplotypes present in sweet cherry (S_1, S_4, S_6, S_9, S_{12}, and S_{13}) have been shown to be present in sour cherry as well. However, three of these *S*-haplotypes (S_1, S_6, and S_{13}) also have non-functional variants in sour cherry that have lost pollen and/or stylar function (Yamane *et al*., 2003*a*; Hauck *et al*., 2006*a*, *b*; Tsukamoto *et al*., 2006). Loss of function in these non-functional *S*-haplotypes was due to structural alterations of the *S-RNase*, *SFB* or *S-RNase* upstream sequences. Sour cherry also possesses *S*-haplotypes (S_{26}, S_{36a}, S_{36b}, S_{36b2}, and S_{36b3}) that were presumably derived from the other species parent, *P. fruticosa*, as these *S*-haplotypes have not been identified in sweet cherry (Hauck *et al*., 2006*b*; T Tsukamoto *et al*., unpublished data). The extensive sour cherry germplasm collection at Michigan State University (Iezzoni, 2005) provides an excellent resource for the identification of previously undiscovered *S*-haplotypes for which information about their functionality would aid in the breeding of SC types. Therefore a germplasm survey was undertaken to search for novel *S*-haplotypes in sour cherry and to determine the functionality of these *S*-haplotypes. Here two new *S*-haplotypes identified in sour cherry are described that are presumed to be contributed by the *P. fruticosa* species parent. The first complete *S-RNase* and *SFB* sequences for a third *S*-haplotype that was previously identified in sour cherry are also reported. Furthermore, genetic segregation data presented demonstrates that all three of these sour cherry *S*-haplotypes are fully functional. In addition, the S_{14}- and S_{16}-haplotypes have been identified in sour cherry. Phylogenetic analyses of *S-RNase* and *SFB* sequences are used to investigate patterns of evolution of *S*-haplotypes in *Prunus*.

Materials and methods

Plant material

One sweet cherry cultivar 'Hedelfingen' (S_3S_5) and 17 sour cherry cultivars were used: 'Cigány 59', 'Crisana', 'Englaise Timpurii', 'Erdi Botermo', 'Erdi Jubileum', 'Erdi Nagygyumolcsu', 'Meteor', 'Montmorency', 'Pandy 38', 'Pandy 114', 'Rheinische Schattenmorelle', 'Surefire', 'Tamaris', 'Tarina', 'Tschernokorka', 'Újfehértói fűrtős', and 'MSU III 18 (12)'. These cultivars were grown at the Michigan State University Clarksville Horticultural Experimental Station, Clarksville, MI, USA and a commercial orchard in Suttons Bay, MI, USA. To determine the inheritance of the *S*-haplotypes, self-pollinated progeny of 'Meteor', 'Montmorency' and 'Tamaris' plus cross-pollinated progeny between 'Újfehértói fűrtős'×'Surefire' were obtained.

DNA isolation

Young unfolded leaves were collected in the spring, frozen in liquid nitrogen, lyophilized, and stored at –20 °C. Genomic DNA was isolated from lyophilized leaves according to the method of Ikeda *et al*. (2004*b*). Extracted leaf DNA was treated with RNase A (Roche, Mannheim, Germany). To genotype the self-pollinated progeny of 'Meteor', 'Montmorency', and 'Tamaris', DNA was extracted from mature seed from which the testa was removed using the procedure of Hauck *et al*. (2006*b*). Extracted seed DNA was treated with RNase A (Roche).

PCR amplification

Total DNA was isolated from the cherry selections and used as template DNA for PCR. PCR procedures were identical to those used by Tao *et al*. (1999). The *S-RNase* gene specific primer set, Pru-C2 and PCE-R (Tao *et al*., 1999; Yamane *et al*., 2001) that correspond to the previously identified C2 and C3 conserved regions (Ushijima *et al*., 1998), respectively, were used. This primer pair can differentiate among most *S-RNase* alleles based on polymorphisms in the length of the second intron in the *Prunus S-RNase*. However, this primer pair cannot amplify S_{35}*-RNase*. EM-PC2consFD and EM-PC5consRD (Sutherland *et al*., 2004) were used to amplify S_{35}*-RNase*. In addition, the primer pair of Pru-C2 and PCE-R cannot differentiate S_{36a} from S_{36b}, S_{36b2} and S_{36b3}. Instead, these variants of the S_{36}-haplotype were differentiated using the following primer pairs for detection of the S_{36a}-haplotype [PcS36ab-F (5′-GCTAGCCAACCACTTTTACG-3') and PcS36a-spR (5′-GAAACCCACATGATACAAACTG-3')] and detection of the S_{36b}-, S_{36b2}-, and S_{36b3}-haplotypes [PcS36ab-F and PcS36b-spR (5′-ATACATTGTAGGCCAGTCTGTG-3')]. PCR products were run on 2% agarose gels and the DNA bands were visualized by

ethidium bromide staining. Furthermore, PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA), and transformed *Escherichia coli* JM109 (Promega). Plasmid DNA was prepared using Wizard *Plus* Minipreps DNA Purification Kit (Promega) and their sequences were determined as described below.

Construction and screening of genomic libraries

Fosmid libraries were constructed using the Copy Control Fosmid library production kit (Epicentre Technologies, Madison, WI, USA). Fosmid libraries from 'Meteor', 'Montmorency', and 'Tamaris' were constructed to clone the *S-RNase* and *SFB* alleles from the *S*-haplotypes being investigated. The fosmid libraries were screened at 60 °C with a mixture of DIG-dUTP-labelled S_6*-RNase* and SFB_6 probes, as previously described (Ushijima *et al*., 2001). The DIG-labelled S_6*-RNase* and SFB_6 probes were obtained by PCR-labelling using the PCR DIG Probe Synthesis Kit (Roche) with Pru-C2 and PCE-R primers (Tao *et al*., 1999; Yamane *et al*., 2001), and SFB-C1F (Ikeda *et al*., 2004*a*) and SFB-C4R primers (Yamane *et al*., 2003*c*). Fosmid DNAs from positive clones were prepared using the Wizard *Plus* Minipreps DNA Purification Kit (Promega). The *S*-haplotype of each positive fosmid clone was determined by PCR with the *S-RNase* consensus primer pair (Pru-C2 and PCE-R). Positive clones were also analysed by PCR with the *SFB* consensus primer pair (SFB-C1F and SFB-C2R) (Ikeda *et al*., 2004*a*) to check if the clone has *SFB*. *S-RNase* and *SFB* allele-specific primer pairs were also used to identify the *S*-haplotypes. For the S_{35}-haplotype in 'Montmorency' ($S_6S_{13m}S_{35}S_{36a}$), positive clones were obtained by using a DIG-dUTP-labelled S_{35}*-RNase* probe. Then more positive clones obtained by hybridization with a mixture of DIG-dUTP-labelled SFB_6 and SFB_{36a} probes were subjected to PCR with the *SFB* consensus primer (SFB-C1F and SFB-C2R) to confirm that the clones contain *SFB*. Then positive clones were analysed by PCR with *SFB* allele-specific (SFB_6, SFB_{13}, and SFB_{36a}/SFB_{36b}) primer pairs: PaSFB6-F and PaSFB6-R (Ikeda *et al*., 2005), DdeS13-F (Tsukamoto *et al*., 2008) and SFB13-spR (Tsukamoto *et al*., 2006), and PcSFB36ab-F (5′-GGCGGTCG-ATCCTGATGAC-3′) and PcSFB36ab-R (5′-TGTCCGATAAC-AGCTCCGG-3′), respectively. The positive clones from which a fragment could be amplified with the primer pair SFB-C1F and SFB-C2R, but not amplified with SFB_6-, SFB_{13}-, and SFB_{36a}/SFB_{36b}-specific primers, were determined to contain SFB_{35} and one positive clone was sequenced.

DNA sequencing

DNA sequencing was carried out by using ABI PRISM 3100 Genetic Analyser at the Michigan State University Research Technology Support Facility. The plasmid clones were sequenced by using SP6 and T7 primers. The fosmid clones were sequenced by primer walking using Pru-T2, Pru-C2, Pru-C2R, PCE-F, PCE-R, Pru-C4R, and Pru-C5 (Tao *et al*., 1999; Yamane *et al*., 2001; Tsukamoto *et al*., 2006) for *S-RNase*, and SFB-C1F, SFB-C2R, SFB-C5F, and FB3R (Ikeda *et al*., 2005) for *SFB*. The EM-PC5consRD primer (Sutherland *et al*., 2004) was also used to sequence S_{35}*-RNase*.

Phylogenetic and variability analysis

For phylogenetic analyses, available nucleotide sequences for *S-RNase* and *SFB* from *Prunus armeniaca*, *P. avium*, *P. cerasus*, *P. domestica*, *P. dulcis*, *P. mume*, *P. salicina*, *P. spinosa*, and *P. tenella S*-haplotypes were assembled (see Fig. 7). Only sequences whose predicted amino acid sequences covered at least 75% of the average length of available complete sequences of S-RNase (227 amino acids) and SFB (375 aminio acids) from the two cherry species (*P. avium* and *P. cerasus*) were included. Nucleotide sequences were translated into deduced amino acid sequences using DAMBE (Xia and Xie, 2001); the amino acid sequences were then aligned using ClustalX (Thompson *et al*., 1997) and adjusted manually. Finally, nucleotide sequences were aligned to the amino acid alignments with DAMBE. Analyses of DNA variability were performed using DnaSP 4.1 (Rozas *et al*., 2003). Phylogenetic analyses of the aligned amino acid and nucleotide sequences based on maximum parsimony were implemented in PAUP* (Swofford, 2002) with heuristic searches using the TBR branch-swapping algorithm and 1000 random taxon addition replicates and maxtrees allowed automatic increases as necessary. Relative support for clades was assessed using 1000 bootstrap replicates with 10 random taxon addition replicates per bootstrap replicate and maxtrees set at 100.

Several approaches were used to test for significant incongruence between the topologies of phylogenetic trees supported by the *SFB* and *S-RNase* data sets, respectively, and all of these approaches were used for both the amino acid and the nucleotide sequences for each gene. First, a combined data set was constructed including only the 49 haplotypes for which sequences of both determinants were available. The partition homogeneity test, implemented in PAUP* with 1000 test replicates and heuristic searches using the TBR branch-swapping algorithm and 10 random taxon addition replicates per test replicate with maxtrees set to 100, was used to test for significant conflict between the *S-RNase* and *SFB* partitions within each data set. Second, each partition was analysed separately and all of the most parsimonious trees were saved to a single tree file. The Kishino-Hasegawa (K-H), Templeton, and winning-sites tests were implemented in PAUP* to test whether or not the topologies produced by the two data partitions were significantly different. Third, because bootstrap analyses showed that many relationships were only weakly supported by each of the two data partitions, constraint trees were constructed in which only groups supported with bootstrap values of 80% or more by each of the data partitions were resolved. Four such constraint trees were constructed; one each for *SFB* and *S-RNase* for the combined amino acid data set and one each for *SFB* and *S-RNase* for the combined nucleotide data set. Each data partition was analysed without constraints and the best trees were saved to a file. Then each data partition was analysed under the constraint corresponding to the well-supported groups from the other partition and the best trees were saved to the same file. The K-H, Templeton, and winning-sites tests were implemented in PAUP* to test whether or not the constrained trees were significantly longer than the unconstrained trees. In order to test whether or not the phylogenetic relationships among *SFB* and *S-RNase* alleles were significantly different from the phylogenetic relationships among species of *Prunus* based on other evidence, each data set was re-analysed with various topological constraints enforced (see Results). For each data set, all of the most parsimonious trees (MPT) from unconstrained and constrained analyses were saved to a single tree file. The K-H, Templeton, and winning-sites tests were implemented in PAUP* to test whether or not the constrained trees were significantly longer than the unconstrained trees.

Results

Identification and cloning of novel S-haplotype genes (S-RNase and SFB) from sour cherry

Genomic PCR with the *S-RNase* consensus primer pair Pru-C2 and PCE-R (Tao *et al*., 1999; Yamane *et al*.,

2001) revealed three amplification products not previously characterized in three sour cherry cultivars (Fig. 1A). Fragments of ~ 480 bp, ~ 420 bp, and ~ 850 bp were identified in 'Englaise Timpurii', 'Meteor', and 'Tamaris', respectively.

The nucleotide sequence of the 481 bp *S-RNase* PCR product from 'Englaise Timpurii' revealed high similarity (99.6%) with the *P. avium* S_{14}*-RNase* partial sequence (GenBank accession no. AJ635277; Sonneveld *et al.*, 2003) and the *P. avium* S_{23}*-RNase* complete sequence (GenBank accession no. AY259114; Wünsch and Hormaza, 2004; the two sequences are identical). The 'Englaise Timpurii' 481 bp *S-RNase* nucleotide sequence differed from the *P. avium* S_{14}*-*/S_{23}*-RNase* by only two base pairs within the second intron (data not shown). Therefore, the 481 bp PCR *S-RNase* product from 'Englaise Timpurii' was considered to be the S_{14}*-RNase*, as the *P. avium* S_{14}- and S_{23}-RNases likely code the same specificity.

BlastN of the 424 bp partial nucleotide sequence of the *S-RNase* PCR product from 'Meteor' revealed high homology (98.3%) with the partial S_{10}*-RNase* of Japanese apricot (*P. mume*) sequence (GenBank accession no. DQ011150; sequence upstream of the C2 conserved region and downstream of the RC4 conserved region is lacking). This novel *S*-haplotype was named S_{33} to follow the previously named functional cherry *S*-haplotypes (S_1 to S_7, S_9, S_{10}, S_{12} to S_{14}, S_{16} to S_{32}) (for a review see Vaughan *et al.*, 2008).

Two S_{33} clones were obtained by screening a 'Meteor' fosmid library. Both clones contained the *S*-haplotype genes, S_{33}*-RNase* and SFB_{33}, and the complete nucleotide sequences of the S_{33}*-RNase* (GenBank accession no. EU054325) and SFB_{33} (GenBank accession no. EU054328) were obtained by sequencing the genes from these two clones. The predicted amino acid sequence of the S_{33}-RNase consisted of 238 residues and was aligned with that of functional S-RNases present in sour cherry (Fig. 2). When the nucleotide sequences of the coding regions of the sour cherry S_{33}*-RNase* and *P. mume* S_{10}*-RNase* were compared, there were four synonymous and one non-synonymous differences. In the second intron, there were four nucleotide differences plus two indels (148 bp and 1 bp long, respectively; see Supplementary Fig. 1 at *JXB* online).

SFB_{33} consisted of 376 amino acid residues that exhibited the characteristic F-box and variability patterns of previously identified SFBs (Fig. 3). S_{33}*-RNase* and SFB_{33} specific primer pairs were designed (Table 1) that amplified the allele specific fragments of 819 bp and 860 bp, respectively (Fig. 4A, B).

BlastN with the 868 bp partial nucleotide sequence of the *S-RNase* PCR product from 'Tamaris' revealed high homology with the S_1*-RNase* obtained from sweet cherry (GenBank accession no. AB031815), the *P. tenella* S_8*-RNase* (GenBank accession no. DQ983367), and partial sequences of the *P. dulcis* S_{11}*-RNase* (GenBank accession

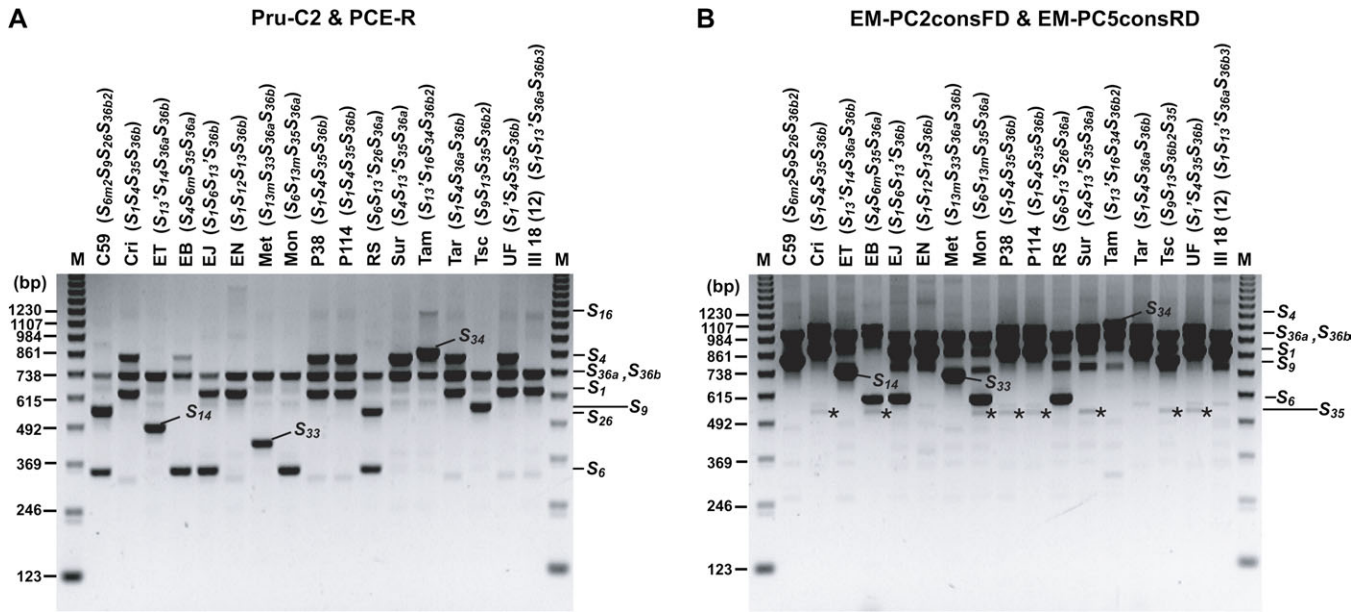

Fig. 1. PCR amplification for *S-RNase* alleles of 17 sour cherry selections. (A) Genomic DNA was amplified by PCR with Pru-C2 (Tao *et al.*, 1999) and PCE-R (Yamane *et al.*, 2001) primer set. (B) Genomic DNA was amplified by PCR with EM-PC2consFD and EM-PC5consRD (Sutherland *et al.*, 2004) primer set. PCR products were separated on 2% agarose gels and detected with ethidium bromide staining. The colour of black and white is inverted in this image. The asterisks indicate the band of PCR product of S_{35}*-RNase*. M, 123 bp DNA ladder (Invitrogen, Carlsbad, CA, USA). Lane abbreviations are: C59, 'Cigány 59'; Cri, 'Crisana'; ET, 'Englaise Timpurii'; EB, 'Erdi Botermo'; EJ, 'Erdi Jubileum'; EN, 'Erdi Nagygyumolcsu'; Met, 'Meteor'; Mon, 'Montmorency'; P38, 'Pandy 38'; P114, 'Pandy 114'; RS, 'Rheinische Schattenmorelle'; Sur, 'Surefire'; Tam, 'Tamaris'; Tar, 'Tarina'; Tsc, 'Tschernokorka'; UF, 'Újfehértói fűrtős'; III 18 (12), 'MSU III 18 (12)'.

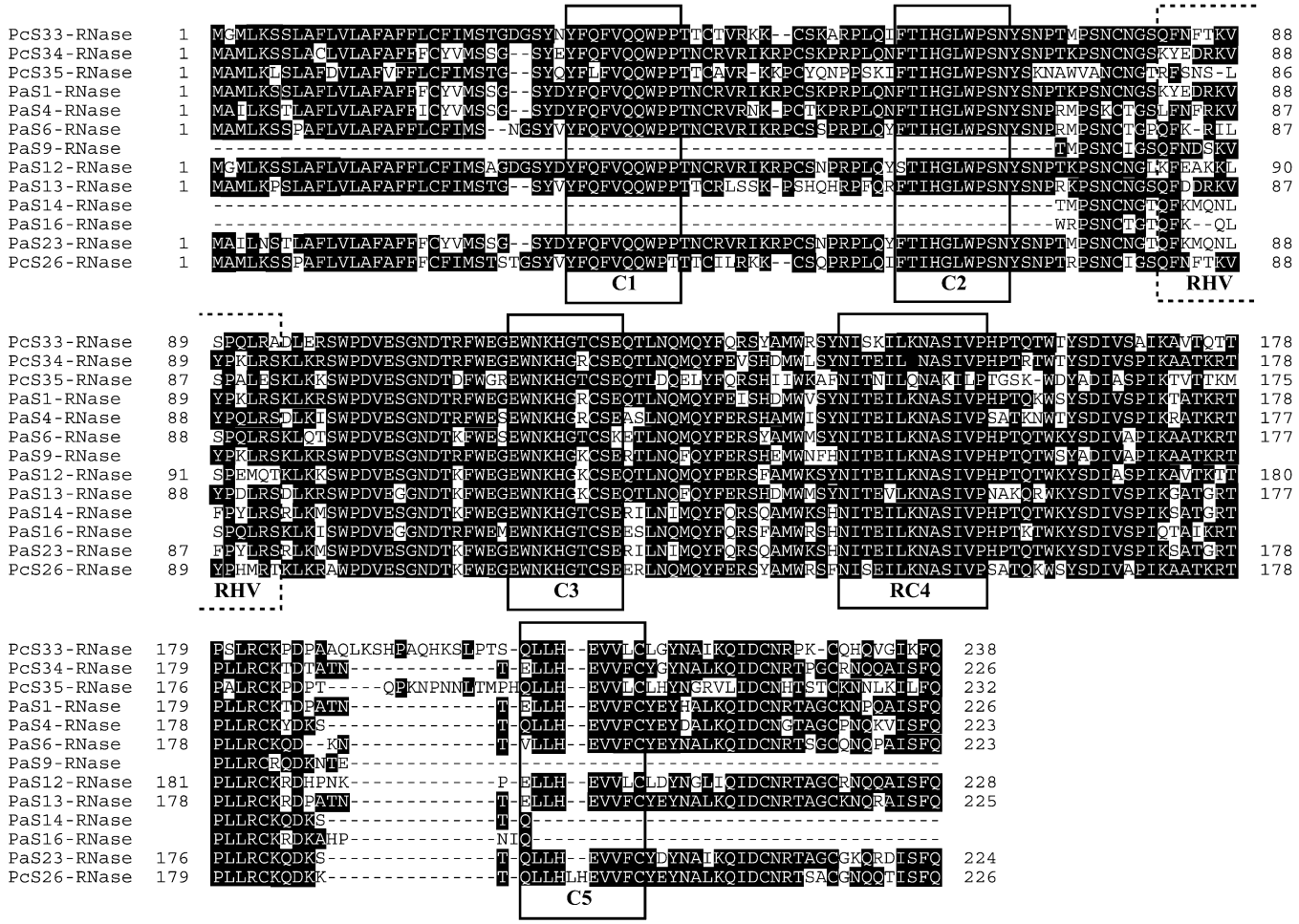

Fig. 2. Amino acid sequence alignment of three novel S-RNases obtained from sour cherry and that of other functional S-RNases from sour cherry. The alignment was generated by DNASIS version 3.5 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). Gaps are marked by dashes. Conserved amino acids are shown on a darkened background. The five conserved regions, C1, C2, C3, RC4, and C5 (Ushijima *et al*., 1998) are marked with solid boxes, and the hypervariable region, RHV (Ushijima *et al*., 1998) reported in the rosaceous S-RNases, is marked with a dotted box. The positions and directions of the four consensus primers used in genomic PCR are indicated by arrows.

no. AM231660) and the *P. domestica* S_5*-RNase* (GenBank accession no. AM746946). This novel *S*-haplotype was named S_{34} (Fig. 5; Table 2). Sour cherry S_{34}*-RNase* falls in this previously described group of *S-RNases* where the sweet cherry S_1*-RNase* was found to be identical to the *P. tenella* S_8*-RNase* and to differ from the *P. dulcis* S_{11}*-RNase* by just one amino acid (Šurbanovski *et al*., 2007).

One S_{34} clone was obtained by screening a 'Tamaris' fosmid library. This clone contained both of the S_{34}*-RNase* and SFB_{34} permitting the complete nucleotide sequencing of the S_{34}*-RNase* (GenBank accession no. EU054326) and SFB_{34} (GenBank accession no. EU054329). The predicted amino acid sequence of S_{34}*-RNase* consisted of 226 residues and was aligned with that of functional S-RNases obtained from sour cherry cultivars (Fig. 2). A comparison of the S_{34}-RNase with a partial sequence of the *P. domestica* S_5-RNase (PdoS$_5$-RNase lacks 30 and 27 amino acids at the N- and C-terminals, respectively) revealed high homology (97%) and only five amino acid differences (Fig. 5). When PcS$_{34}$-RNase was compared with *P. avium* S_1-RNase, 15 amino acid differences were identified (Fig. 5). Nevertheless, the sweet cherry S_1*-RNase* specific primer pair (Sonneveld *et al*., 2001) amplified a product for the S_{34}*-RNase*. However, the amplification product from the S_{34}*-RNase* was the expected size of ~850 bp instead of 615 bp as for the *P. avium* S_1*-RNase* (data not shown). The second intron of the S_{34}*-RNase* is 238 bp longer than that of the *P. avium* S_1*-RNase* (GenBank accession nos AB031815 and AB028153; see Supplementary Fig. 2 at *JXB* online).

SFB$_{34}$ was composed of 376 amino acid residues that exhibited the characteristic variability patterns of previously identified SFBs (Fig. 3). Due to the high homology of the S$_{34}$-RNase with the *P. avium* S$_1$-RNase, an alignment was performed with their respective SFBs. The predicted amino acid sequence of *P. cerasus* SFB$_{34}$

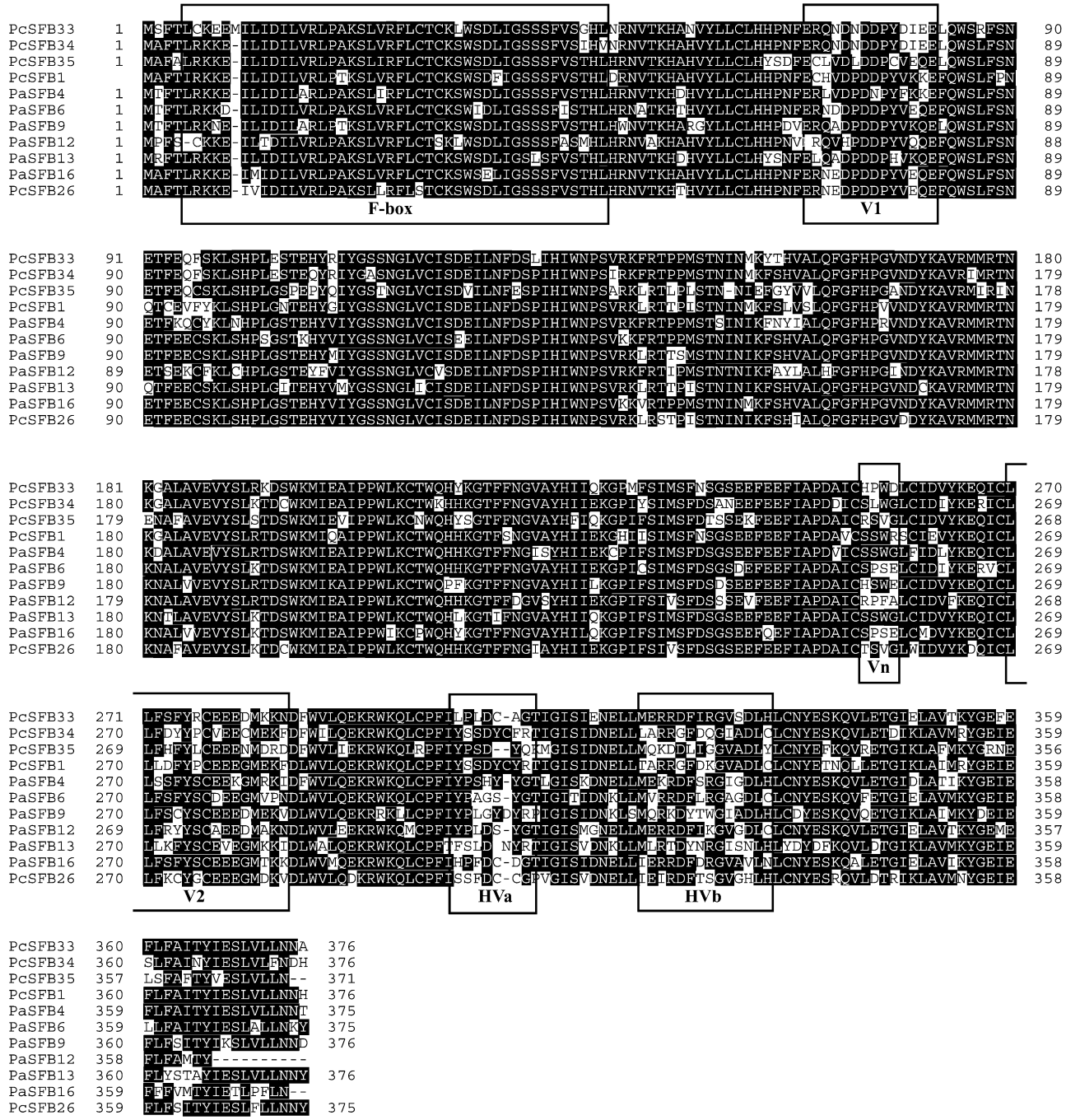

Fig. 3. Amino acid sequence alignment of three novel SFBs obtained from sour cherry and that of other functional SFBs from sour cherry. The alignment was generated by DNASIS version 3.5 (Hitachi Software Engineering Co. Ltd., Tokyo, Japan). Gaps are marked by dashes. Conserved amino acids are shown on a darkened background. The locations of the F-box motif, V1, V2, HVa, and HVb (Ikeda *et al*., 2004*a*), and Vn (Nunes *et al*., 2006) are indicated by solid boxes.

was aligned with three cherry SFB_1 sequences: (i) the *P. cerasus* SFB_1 obtained from 'Pandy 114' (GenBank accession no. DQ827715), (ii) the *P. avium* SFB_1 cloned from 'Skeena' (GenBank accession no. AY805048), and (iii) the *P. avium* SFB_1 sequence obtained from 'Seneca' (GenBank accession no. AB111518) and *P. dulcis* SFB_{11}, and *P. tenella* SFB_8 (Fig. 6). The *P. cerasus* 'Pandy 114' SFB_1 and *P. avium* cv. 'Skeena' SFB_1 sequences are identical, however the 'Skeena' sequence is not complete as 10 amino acid residues at the C-terminal are not determined. The amino acid sequence of the 'Seneca' SFB_1 is slightly different from that of SFB_1 of 'Pandy 114' and 'Skeena' (Fig. 6). Unlike their S-RNases, the predicted amino acid sequences for the sour cherry SFB_{34}

Table 1. *Preferred PCR primer pairs to amplify S-RNase and SFB alleles of S_{33}-, S_{34}-, and S_{35}-haplotypes identified in sour cherry*

Target gene	Primer name	Primer sequence 5′ → 3′	Annealing temperature (°C)	Extension time (s)	Product size (bp)[a]
S_{33}-RNase	PcS33-F	CACAGTTCGCAAGAAATGC	66	60	819
	PcS33-R	ATGTTGGCATTTTGGTCGG			
SFB_{33}	PcSFB33-F	TCATTCACACTATGTAAGGAAGAAATG	63	60	860
	PcSFB33-R	CAAAAGTCATTTTTTTTCATGTCC			
S_{34}-RNase	PcS34-F	GTAATTGCAATGGGTCAAAATATGAG	66	60	898
	PcS34-R	CAGCCACATGTCATGGGATACT			
SFB_{34}	PcSFB34-F	TGTTAGCATACACGTTAACAGG	66	60	714
	PcSFB34-R	CACTCCTCCACACAAGGATAATAGTC			
S_{35}-RNase	PcS35-F	GACCCGATTTAGCAATAGTTTG	66	60	435
	PcS35-R	GAGGCATCGTCAAGTTGTTAG			
SFB_{35}	PcSFB35-F	ACGAATCAACGAAAATGCTTTC	66	60	557
	PcSFB35-R	ATGCAAACGATAATTCATTGCG			

[a] Product size is based on the use of genomic DNA as a template.

and SFB_1 differed by 69 amino acids (Fig. 6) and shared only 81.6% identity (Table 2). Similarly, the amino acid sequence of the *P. cerasus* SFB_{34} had only 81.0% homology with that of the identical *P. tenella* SFB_8 and *P. dulcis* SFB_{11}. By comparison, the *P. cerasus* SFB_{34} and the partial sequence of the *P. domestica* SFB_5 (GenBank accession number. AM746955; $PdoSFB_5$ is lacking 12 and 33 amino acid residues at the N- and C-terminals, respectively) differed at 15 amino acid residues (95.5% homology), compared with 69 different residues between SFB_{34} and SFB_1 (Fig. 6).

To compare the S_{34}- and S_1-haplotypes further, a partial sequence of the intergenic region between *S-RNase* and *SFB* of *P. cerasus* S_{34} was obtained. However, the intergenic sequence for the S_{34}-haplotype was extremely divergent compared with that from the *P. avium* S_1, *P. dulcis* S_{11}, and *P. tenella* S_8 and did not permit a conclusive alignment (see Supplementary Fig. 3 at *JXB* online).

To aid in the confirmation of the S_{34}-haplotype in future germplasm surveys, *S_{34}-RNase* and *SFB_{34}* specific primer pairs were designed (Table 1) that amplified allele specific fragments of expected size (898 bp and 714 bp, respectively) (Fig. 4C, D). The *S_1-RNase* specific primer pair (Sonneveld *et al*., 2001) amplified fragments for the *S_1-RNase* (~615 bp) and *S_{34}-RNase* (~850 bp) (data not shown), but the *S_{34}-RNase* specific primer pair only amplified a product from the *S_{34}-RNase*, not the *S_1-RNase* (Fig. 4C).

To identify all the *S*-haplotypes in 'Tamaris', genomic PCR was performed with all available *S-RNase* specific primer pairs. A fragment of ~ 680 bp was amplified with the *S_{16}-RNase* specific primer pair (Sonneveld *et al*., 2003). A comparison of the nucleotide sequence from the *S_{16}-RNase* PCR product from 'Tamaris' with the *P. avium* *S_{16}-RNase* revealed only one base pair difference in the second intron. Therefore 'Tamaris' was considered to have the S_{16}-haplotype.

Identification and cloning of the S_{35}-RNase and SFB_{35}

Previously an *S*-haplotype survey in sour cherry cultivars was carried out using RFLP analysis, and *S-RNase* based PCR was also carried out using the consensus *S-RNase* gene-specific primer set, Pru-C2 and PCE-R (Tao *et al*., 1999; Yamane *et al*., 2001). Using these two approaches, only three different *S*-haplotypes were characterized in many cultivars, including the landrace sour cherry cultivar 'Pandy' (syn. 'Köröser', 'Crisana') and 'Montmorency'. Yet, genetic studies with many of these cultivars led to the conclusion that none of the three *S*-haplotypes identified in each cultivar was present in a double dose (Hauck *et al*., 2006*b*). For example, the cultivar 'Újfehértói fűrtős' has the *S*-haplotypes S_1', S_4, and S_{36b} of which S_1' and S_{36b} are non-functional. Therefore, it is possible that 'Újfehértói fűrtős' could contain two copies for either of these two non-functional *S*-haplotypes. However, our genetic segregation data was consistent in rejecting this hypothesis (Hauck *et al*., 2006*a*, *b*). Therefore, it is postulated that these cultivars contained a fourth allele and termed it S_{null} as it was not possible to resolve this fourth allele on a Southern blot with either *S-RNase* or *SFB* probes.

Bošković *et al*. (2006) reported the presence of a different *S-RNase*, S_D, in 'Köröser', 'Montmorency', and 'Bruine Waalse' using a different *S-RNase* consensus primer pair (EM-PC2consFD and EM-PC5consRD). Therefore, this alternate primer pair was used to test the possibility that S_D might be the S_{null} allele. Using this primer pair, a ~ 530 bp amplification product was amplified in eight selections ('Crisana', 'Erdi Botermo', 'Montmorency', 'Pandy 38', 'Pandy 114', 'Surefire', 'Tschernokorka', and 'Újfehértói fűrtős'; Fig. 1B), that had a similar size to the *S_D-RNase* (Bošković *et al*., 2006). The 527 bp *S-RNase* PCR products amplified with the EM-PC2consFD and EM-PC5consRD primers from 'Crisana', 'Pandy 114', and 'Újfehértói fűrtős' were cloned and sequenced and the nucleotide sequences were

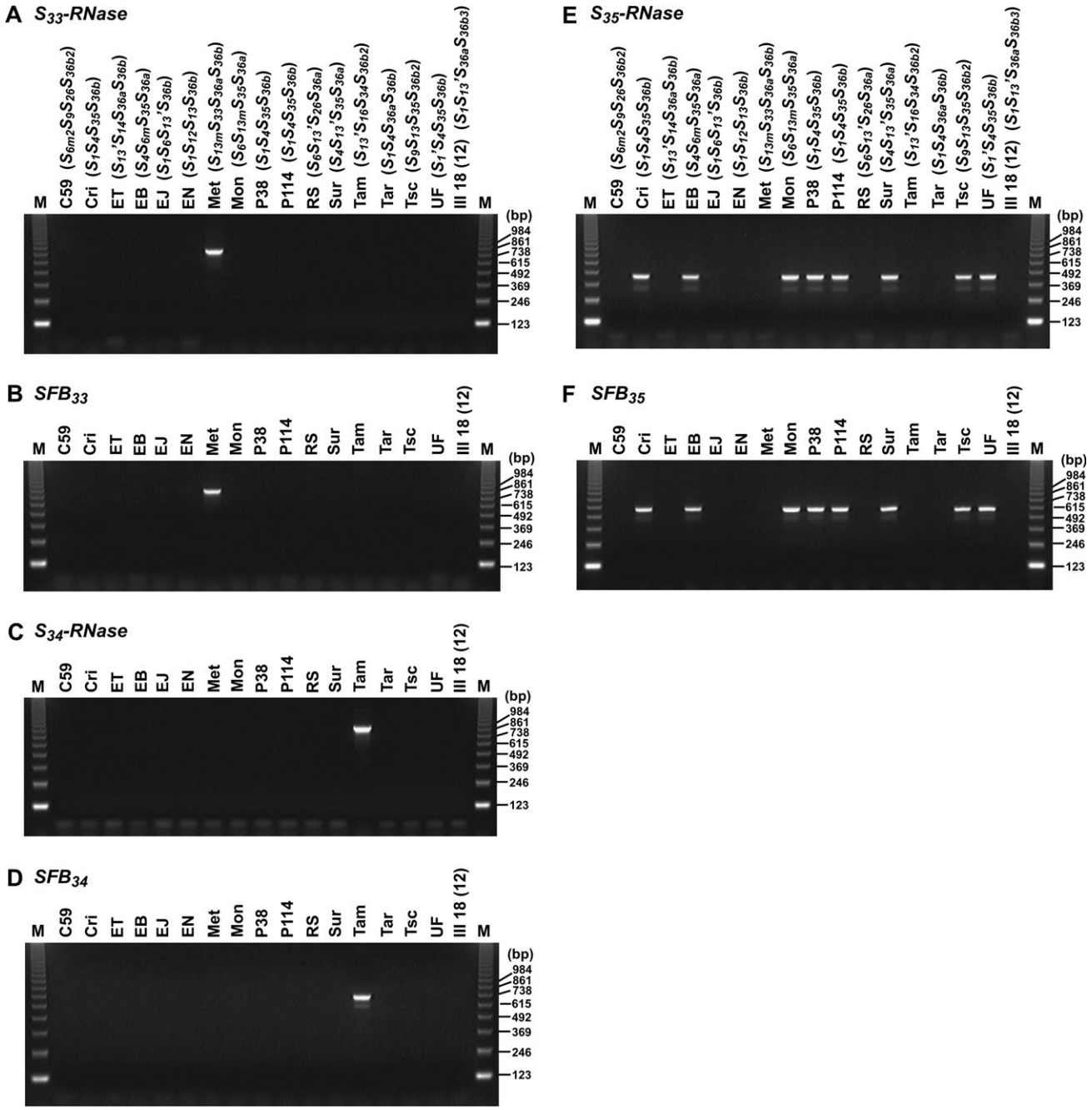

Fig. 4. PCR amplification with S_{33}-, S_{34}-, and S_{35}-allele specific primer pair for *S-RNase* and *SFB* in 17 sour cherry selections. PCR products were separated on 2% agarose gel and detected with ethidium bromide staining. M, 123 bp DNA ladder (Invitrogen, Carlsbad, CA, USA). Lane abbreviations are: C59, 'Cigány 59'; Cri, 'Crisana'; ET, 'Englaise Timpurii'; EB, 'Erdi Botermo'; EJ, 'Erdi Jubileum'; EN, 'Erdi Nagygyumolcsu'; Met, 'Meteor'; Mon, 'Montmorency'; P38, 'Pandy 38'; P114, 'Pandy 114'; RS, 'Rheinische Schattenmorelle'; Sur, 'Surefire'; Tam, 'Tamaris'; Tar, 'Tarina'; Tsc, 'Tschernokorka'; UF, 'Újfehértói fűrtős'; III 18 (12), 'MSU III 18 (12)'.

identical. Unfortunately, these nucleotide sequences could not be compared with that of S_D-*RNase* since the latter is not available in GenBank. Nevertheless, a comparison using the amino acid sequence available in Fig. 2 of Bošković *et al*. (2006) revealed that our sequence differed from S_D-RNase in two regions (see Supplementary Fig. 4 at *JXB* online). One region is just before the first intron. The S_D-RNase has two additional glycine residues not present in our S-RNase sequence. The other region is the RHV region just after the second intron. The reasons for these discrepancies are not known. However, as the validity of our full length *S-RNase* sequences were verified multiple times from fosmid clones from three different genotypes (see below), and genetically verified

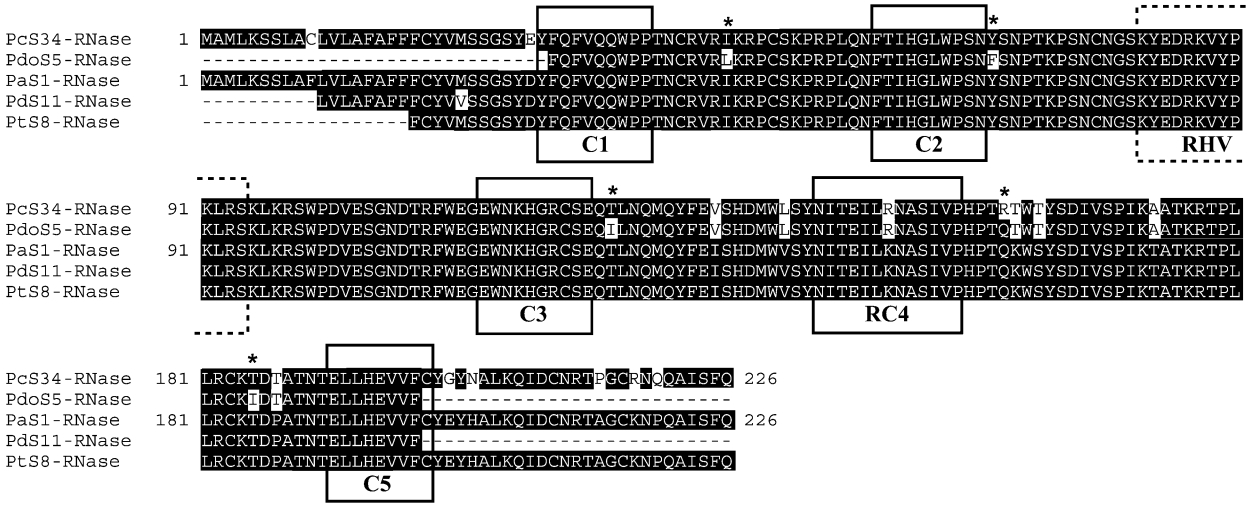

Fig. 5. The deduced amino acid sequence alignment for the *Prunus cerasus* S_{34}-RNase (PcS34-RNase), *P. domestica* S_5-RNase (PdoS5-RNase), *P. avium* S_1-RNase (PaS1-RNase), *P. dulcis* S_{11}-RNase (PdS11-RNase), and *P. tenella* S_8-RNase (PtS8-RNase). The asterisks indicate the five amino acid residues that are different between *P. cerasus* S_{34}-RNase and *P. domestica* S_5-RNase. Conserved nucleotides are shown on a darkened background. The five conserved regions, C1, C2, C3, RC4, and C5 (Ushijima *et al*., 1998) are marked with solid boxes, and the hypervariable region, RHV (Ushijima *et al*., 1998) reported in the rosaceous S-RNases, is marked with a dotted box.

Table 2. *Identities of the derived amino acid sequences among the cherry S-locus genes whose complete sequence are available*

The upper half presents amino acid sequence identities (%) between cherry SFBs; the lower half between the S-RNases. The sequence identity in a parenthesis was calculated by using partial amino acid sequence. n.a. means that sequence data is not available in GenBank. Pa, *Prunus avium*; Pc, *P. cerasus*. The sequences used are as follows; $PaSFB_1$ (AY805048 and DQ983372), $PaSFB_2$ (AB111519), $PaSFB_3$ (AB096857), $PaSFB_4$ (AB111521), $PaSFB_5$ (AB111520), $PaSFB_6$ (AB096858), $PaSFB_7$ (EU035976), $PaSFB_9$ (DQ422809), $PaSFB_{12}$ (AY805054), $PaSFB_{13}$ (DQ385844), $PcSFB_{26}$ (EU035977), $PcSFB_{33}$ (EU054328), $PcSFB_{34}$ (EU054329), $PcSFB_{35}$ (EU054330), PaS_1-RNase (AB028153), PaS_2-RNase (AB010304), PaS_3-RNase (AB010306), PaS_4-RNase (AB028154), PaS_5-RNase (AJ298314), PaS_6-RNase (AB010305), PaS_7-RNase (EU035974), PaS_9-RNase (AJ635270), PaS_{12}-RNase (AY259115), PaS_{13}-RNase (DQ385842), PaS_{23}-RNase (AY259114), PaS_{24}-RNase (AY259112), PaS_{25}-RNase (AY259113), PcS_{26}-RNase (EU035975), PcS_{33}-RNase (EU054325), PcS_{34}-RNase (EU054326), and PcS_{35}-RNase (EU054327).

	PaS_1	PaS_2	PaS_3	PaS_4	PaS_5	PaS_6	PaS_7	PaS_9	PaS_{12}	PaS_{13}	PaS_{23}	PaS_{24}	PaS_{25}	PcS_{26}	PcS_{33}	PcS_{34}	PcS_{35}
PaS_1	–	76.6	76.8	77.6	75.3	76.0	75.4	77.1	(73.2)	77.1	n.a.	n.a.	n.a.	77.3	76.3	81.6	70.6
PaS_2	(84.3)	–	77.5	80.3	79.8	80.6	76.8	79.5	(76.5)	80.4	n.a.	n.a.	n.a.	80.4	80.4	77.3	73.9
PaS_3	73.9	(72.6)	–	77.6	77.5	80.1	78.3	78.1	(75.8)	75.5	n.a.	n.a.	n.a.	77.9	82.4	82.4	73.8
PaS_4	81.4	(79.8)	69.4	–	78.7	82.1	78.1	79.7	(79.1)	79.2	n.a.	n.a.	n.a.	81.0	80.9	78.7	74.8
PaS_5	75.4	(73.6)	69.3	74.0	–	76.9	80.5	77.9	(75.7)	76.7	n.a.	n.a.	n.a.	77.7	77.7	73.9	72.3
PaS_6	80.1	(85.1)	73.5	78.6	73.2	–	77.3	80.5	(79.9)	79.3	n.a.	n.a.	n.a.	81.1	80.5	79.9	73.7
PaS_7	77.9	(82.2)	70.4	77.7	78.5	81.7	–	79.4	(74.6)	76.2	n.a.	n.a.	n.a.	79.4	76.8	75.1	71.9
PaS_9	(80.5)	(79.7)	(70.2)	(75.2)	(73.5)	(78.4)	(74.6)	–	(76.4)	81.3	n.a.	n.a.	n.a.	79.5	79.8	77.1	74.6
PaS_{12}	79.4	(82.4)	73.5	71.1	70.4	78.9	79.8	(73.7)	–	(73.9)	n.a.	n.a.	n.a.	(76.2)	(79.5)	(74.3)	(72.1)
PaS_{13}	81.9	(79.0)	72.1	76.0	71.4	77.9	74.3	(76.7)	74.1	–	n.a.	n.a.	n.a.	80.9	77.7	76.3	73.5
PaS_{23}	79.2	(81.7)	71.3	78.6	75.4	81.3	79.5	(75.4)	76.8	73.5	–	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PaS_{24}	75.7	(80.3)	72.9	76.2	69.2	92.8	77.2	(77.6)	74.1	77.8	75.9	–	n.a.	n.a.	n.a.	n.a.	n.a.
PaS_{25}	78.8	(76.8)	67.9	72.5	73.7	73.7	73.7	(79.5)	75.6	69.1	75.4	69.1	–	n.a.	n.a.	n.a.	n.a.
PcS_{26}	75.9	(78.1)	69.3	75.3	71.9	78.9	78.5	(78.8)	72.4	75.3	73.7	78.0	68.5	–	78.1	77.9	74.3
PcS_{33}	68.9	(81.1)	71.0	74.5	68.2	75.6	77.2	(72.0)	71.8	67.9	73.1	76.6	73.4	72.0	–	82.7	74.1
PcS_{34}	93.4	(84.8)	72.6	80.1	73.2	80.1	78.8	(79.7)	79.4	79.6	78.3	75.7	78.4	74.6	70.1	–	72.7
PcS_{35}	58.3	(57.1)	58.3	54.7	59.4	58.1	59.1	(58.6)	61.2	59.0	56.6	57.9	59.3	56.8	59.6	55.7	–

in inheritance studies (see below), it has been tentatively postulated that our sequence indeed represents the previously identified S_D-haplotype. This *S*-haplotype is named S_{35}.

To clone the *S_{35}-RNase* and *SFB_{35}*, a fosmid library of 'Montmorency' ($S_6S_{13m}S_{35}S_{36a}$) was first screened using a probe of DIG-dUTP-labelled with a 527 bp *S_{35}-RNase* fragment amplified by EM-PC2consFD and EM-PC5consRD from 'Crisana'. Sixteen positive clones were obtained. Among them, two clones (Mon37 and Mon46) were shown to contain the *S_{35}-RNase* since an ~530 bp fragment was amplified by PCR with the *S-RNase* consensus primer pair (EM-PC2consFD and EM-PC5consRD) but no fragment was obtained with the other *S-RNase* consensus primer pair (Pru-C2 and PCE-R). Unfortunately these two clones did not contain the *SFB_{35}*

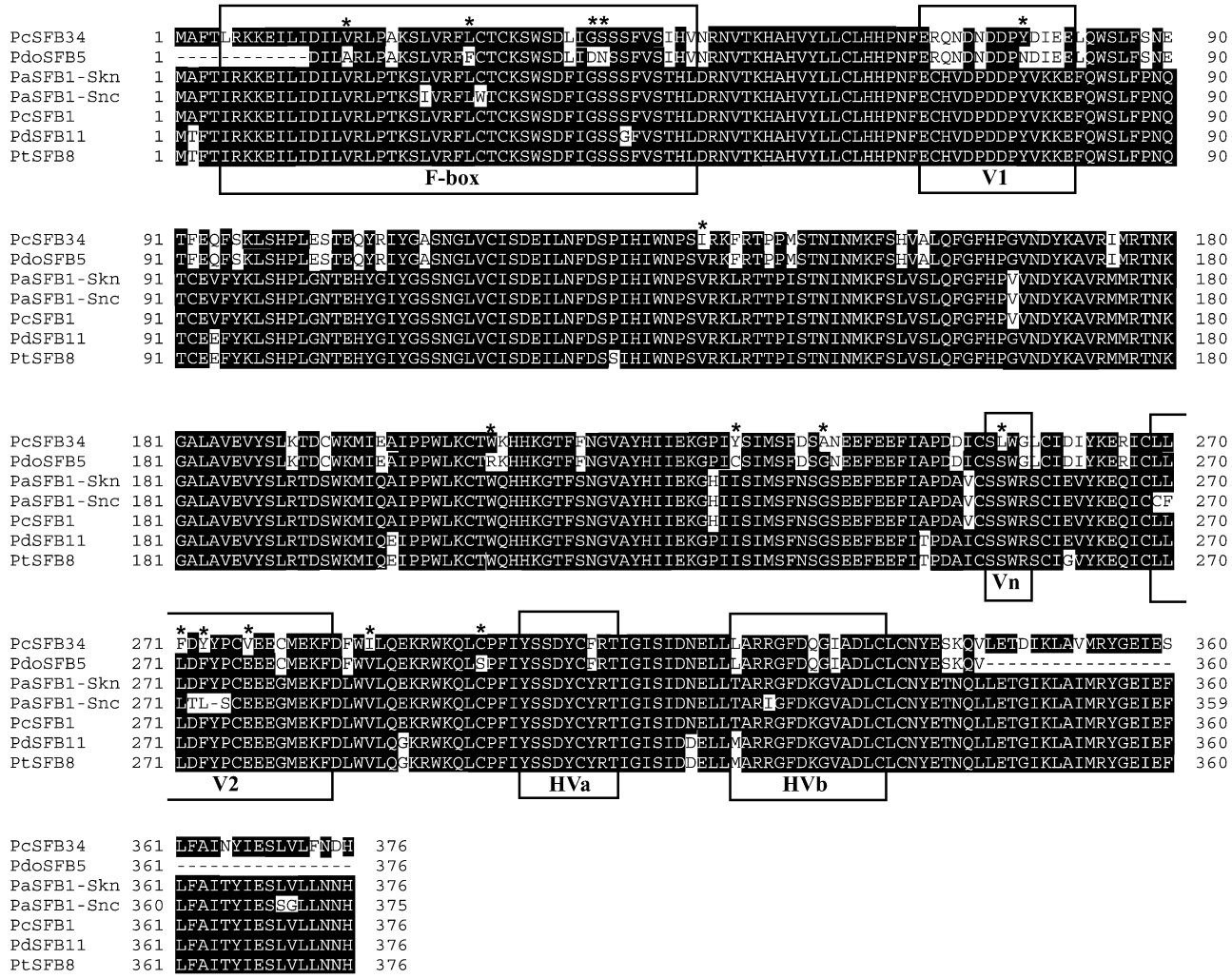

Fig. 6. The deduced amino acid sequence alignment for the *Prunus cerasus* SFB_{34} (PcSFB34), *P. domestica* SFB_5 (PdoSFB5), *P. avium* SFB_1 obtained from 'Skeena' (PaSFB1-Skn), *P. avium* SFB_1 obtained from 'Seneca' (PaSFB1-Snc), *P. cerasus* SFB_1 obtained from 'Pandy 114' (PcSFB1), *P. tenella* SFB_8 (PtSFB8), and *P. dulcis* SFB_{11} (PdSFB11). The asterisks indicate the 15 amino acid residues that are different between *P. cerasus* SFB_{34} and *P. domestica* SFB_5. Conserved nucleotides are shown on a darkened background. The locations of the F-box motif, V1, V2, HVa, and HVb (Ikeda *et al*., 2004*a*), and Vn (Nunes *et al*., 2006) are indicated by solid boxes.

since the *SFB* band was not amplified by PCR with the *SFB* consensus primer pair (SFB-C1F and SFB-C2R). Next the 'Montmorency' fosmid library was screened again with a mixture of DIG-dUTP-labelled SFB_6 and SFB_{36a} probes at lower stringency (55 °C) and obtained 25 positive clones. Among them, six clones (Mon64, Mon71, Mon74, Mon76, Mon122, and Mon132) were considered to contain the novel *SFB* since these six clones amplified a *SFB* fragment by PCR with the *SFB* consensus primer (SFB-C1F and SFB-C2R) but not with allele specific primer pairs for SFB_6, SFB_{13}, and SFB_{36a}/SFB_{36b}. This novel *SFB* was named SFB_{35}. These six clones were also analysed by PCR with the *S-RNase* primer pair EM-PC2consFD and EM-PC5consRD, but no fragment was amplified from any of the six clones. Therefore these six clones were considered to contain SFB_{35} but not to contain S_{35}-*RNase*. This suggests that the intergenic distance between the S_{35}-*RNase* and SFB_{35} is larger than that observed in the majority of cherry *S*-haplotypes (380 bp to 38 kb) identified to date (Ikeda *et al*., 2005) as the average insert size of our fosmid clones was ~ 40 kb. Mon37 and Mon64 were sequenced to determine the complete nucleotide sequences of the S_{35}-*RNase* (GenBank accession no. EU054327) and SFB_{35} (GenBank accession no. EU054330), respectively. The S_{35}-*RNase*, which consists of 232 amino acid residues (Fig. 2), is very different from other S-RNases identified in cherry. The second intron of S_{35}-*RNase* is extremely short, consisting of only 82 bp (see Supplementary Fig. 4 at *JXB* online). The *S-RNase* consensus primer pair (Pru-C2 and PCE-R) could not amplify S_{35}-*RNase* because the Pru-C2 primer was designed based on the amino acid

sequence LWPSNYSN and the S_{35}-RNase has LWPSNYSK. The PCE-R primer was designed based on the amino acid sequence EXEWNK, but the deduced amino acid sequence for S_{35}-RNase is GREWNK.

SFB_{35} is composed of 371 amino acid residues that exhibited the characteristic variability patterns of previously identified SFBs (Fig. 3). Using these sequences, *S_{35}-RNase* and *SFB_{35}* specific primers were designed (Table 1). The *S_{35}-RNase* specific primer pair amplified a fragment of the expected size (435 bp) in all eight selections from which the 527 bp PCR product was amplified with the EM-PC2consFD and EM-PC5consRD primer pair (Fig. 4E). The *SFB_{35}* specific primer pair also amplified fragments of the expected size (557 bp) in all eight selections that had a 527 bp fragment following amplification using the EM-PC2consFD and EM-PC5consRD primer pair (Fig. 4F). It was further confirmed that the ~560 bp fragment was amplified by the *SFB_{35}* specific primer pair in all six fosmid clones (Mon64, Mon71, Mon74, Mon76, Mon122, and Mon132) (data not shown).

S-*haplotype functionality*

The functionality of the *S*-haplotypes identified in sour cherry was tested using populations derived from self- and cross-pollination. In the parents of these populations the *S*-haplotype being tested would segregate in both the eggs and pollen grains in an expected 1:1 ratio assuming that these gametes are viable and equally probable of occurring in a successful gamete. Therefore, if the *S*-haplotype is functional in the style and pollen, the pollen that contains that *S*-haplotype would be incompatible and the ratio of that *S*-haplotype would be 1:1 to represent its expected ratio in the eggs. However, if the *S*-haplotype being tested has lost either pollen or stylar function, the pollen carrying that *S*-haplotype would be compatible and that *S*-haplotype would be expected to be present in the progeny in a 3:1 ratio representing the additional contribution of the pollen *S*-haplotype. Using this strategy, multiple functional and non-functional *S*-haplotypes were previously identified in sour cherry (Hauck *et al*., 2006*b*).

To determine if the *S_{33}*-haplotype in 'Meteor' was fully functional, the self-pollinated progeny of 'Meteor' were genotyped for their *S*-haplotypes. The progeny segregation ratio for *S_{33}* fit the expected 1:1 ratio and rejected the 3:1 ratio indicating that *S_{33}* is a functional *S*-haplotype (Table 3). Self-pollinated progeny of 'Tamaris' also segregated according to a 1:1 rejecting the 3:1 ratio for the *S_{34}*-haplotype indicating that this *S*-haplotype is functional (Table 3). As 'Tamaris' is the first report of the presence of the sweet cherry *S_{16}*-haplotype in sour cherry, the functionality of this *S*-haplotype was also tested. *S_{16}* segregated according to a 1:1 ratio indicating that the *S_{16}*-haplotype is also functional.

S_{35} was identified in several cultivars with diverse geographic origin; therefore, three different cultivars with diverse origins were used to test the functionality of *S_{35}*. Segregation of *S_{35}* in the self-pollinated progeny of 'Montmorency' fit a 1:1 ratio and rejected the 3:1 ratio indicating that *S_{35}* is a functional *S*-haplotype in this cultivar (Table 3). As *S_{35}* is also present in 'Újfehértói fűrtős' and 'Surefire' it allowed us to test whether the *S_{35}* containing pollen from 'Surefire' is compatible in 'Újfehértói fűrtős' styles. The segregation of *S_{35}* in the progeny from this cross segregated according to the 1:1 ratio, rejecting the 3:1 ratio confirming that *S_{35}* is a functional *S*-haplotype.

'Montmorency', 'Újfehértói fűrtős', and 'Surefire' had previously been determined to have the *S_{null}*-haplotype as our genetic data predicted the presence of a fourth *S*-haplotype (Hauck *et al*., 2006*b*). To test the hypothesis that *S_{null}* is the newly identified *S_{35}*, the progeny from self-pollinated 'Montmorency' and 'Újfehértói fűrtős'×'Surefire' were *S*-genotyped with the addition of

Table 3. *Progeny segregation of the S_{16}-, S_{33}-, S_{34}- and S_{35}-haplotypes to test the functionality of each S-haplotype*

Population		*S*-haplotype segregation				
S-genotype(s) of parent(s)	No. of progeny observed	*S*-haplotype tested	Expected ratio[a]	Observed ratio + : −	Chi square	*P*-value
'Meteor' (*$S_{13m}S_{33}S_{36a}S_{36b}$*) self-pollinated	83	*S_{33}*	1:1	43 : 40	0.108	0.7419
			3:1	43 : 40	28.81	<0.0001
'Tamaris' (*$S_{13}'S_{16}S_{34}S_{36b2}$*) self-pollinated	37	*S_{16}*	1:1	20 : 17	0.243	0.6219
			3:1	20 : 17	8.658	0.0033
		S_{34}	1:1	20 : 17	0.243	0.6219
			3:1	20 : 17	8.658	0.0033
'Montmorency' (*$S_6S_{13m}S_{35}S_{36a}$*) self-pollinated	81	*S_{35}*	1:1	43 : 38	0.309	0.5785
			3:1	43 : 38	20.74	<0.0001
'Újfehértói fűrtős' (*$S_1'S_4S_{35}S_{36b}$*) ×'Surefire' (*$S_4S_{13}'S_{35}S_{36a}$*)	116	*S_{35}*	1:1	64 : 52	1.241	0.2652
			3:1	64 : 52	24.32	<0.0001

[a] A 1:1 ratio is expected if the *S*-haplotype is fully functional. Therefore the progeny would only be able to obtain this *S*-haplotype from the egg. A 3:1 ratio is excepted if the *S*-haplotype is fully functional as this *S*-haplotype could also be contributed to the progeny through the pollen.

the S_{35}. In all cases S_{35} co-segregated with the previous prediction of S_{null} (data not presented) confirming that S_{35} is the fourth *S*-haplotype in these selections.

Phylogenetic analyses

Phylogenetic analyses of amino acid and nucleotide sequences of *S-RNase* and *SFB* sequences from eight species of *Prunus* (Fig. 7) produced trees in which the closest relatives of many alleles were alleles from other species. This pattern, first reported for Solanaceae species, was named trans-specific evolution by Richman *et al.* (1996). In *Prunus* this pattern has been described before (see references in Vieira *et al.*, 2008), but in contrast with the observation made for the Solanaceae species, in this genus trans-specific evolution cannot be taken as evidence for the very old age of alleles (Vieira *et al.*, 2008). Although this pattern has been known for more than a decade, no proper phylogenetic analyses have so far been performed to show conclusively that this pattern is not simply due to lack of phylogenetic resolution. In *Prunus*, bootstrap support values are generally weak. Nevertheless, in no case were all the alleles of any one gene from any one species supported as monophyletic.

Phylogenetic analyses of nucleotide sequences of both *S-RNase* and *SFB* data resolved the *P. cerasus* S_{35} along with the *P. dulcis* S_a as highly divergent from the remaining *S*-haplotypes (Fig. 7). Therefore, S_{35} was identified as not only divergent from other cherry *S*-haplotypes but also divergent from other *Prunus* *S*-haplotypes. For the *P. cerasus* S_{33} and *P. cerasus* S_{34}, as well as for the alleles from several other *S*-haplotypes, the resolved relationships were different in the two data sets (Fig. 7). All data sets strongly supported the sister relationship between *P. cerasus* S_{34} and *P. domestica* S_5. Both the amino acid and the nucleotide sequence data resolved the *S-RNases* of the last two haplotypes as sister to a clade including the *P. avium* S_1*-RNase*, the *P. dulcis* S_{11}*-RNase*, and the *P. tenella* S_8*-RNase*. The *SFB* data did not, however, show this relationship, and the position of *P. cerasus* SFB_{34} plus *P. domestica* SFB_5 was very weakly supported. The *P. cerasus* S_{33}*-RNase* was weakly supported as sister to *P. mume* S_1*-RNase* and *P. spinosa* S_{10}*-RNase*; however, *P. cerasus* SFB_{33} was resolved, again with very weak support, as sister to the clade of *P. avium* SFB_3, *P. cerasus* SFB_{34}, and *P. domestica* SFB_5. The relationships resolved by the amino acid sequence data for both genes (not shown) differed in some details from those resolved by the nucleotide sequence data, especially for the more weakly supported relationships.

Results of the Partition Homogeneity test revealed strong differences in phylogenetic signal between the *S-RNase* and the *SFB* nucleotide data sets as well as between the amino acid data sets for the two genes (P=0.001 for all tests). Tests were carried out for significant differences in the tree topologies between the *S-RNase* and *SFB* data for both nucleotides and amino acids using the Templeton, K–H, and winning-sites tests, as described above. For all cases in which data from one gene were constrained to give topologies identical to those found to be optimal for the other gene, the resulting trees were significantly longer than unconstrained trees (P <0.0001 for all tests). In contrast, when data from one gene were constrained to resolve only the groups supported with bootstrap values of 80% or greater by the other gene, the results were not significant for any data set or any test.

Because resolutions of relationships among the *Prunus* *S-RNase* and *SFB* alleles were generally weak, the possibility was tested that topologies consistent with one or more constraints concordant with our understanding of organismal relationships based on other data (Bortiri *et al.*, 2001) were not significantly worse than the best (most parsimonious) topologies resolved for the two data sets containing just the *Prunus* sequences. Constraint trees with three distinct topologies were used to test these hypotheses. In the first topology, the 'species' constraint, all representatives of each species were constrained to form a monophyletic group, but no restrictions were placed on the relationships among species. In the second topology, the 'subgenera' constraint, each of several groups of 2–4 species (*P. avium*/*P. cerasus*, *P. armeniaca*/*P. mume*, *P. dulcis*/*P. tenella*, and *P. cerasifera*/*P. domestica*/*P. salicina*/*P. spinosa*) was constrained as monophyletic, but no other restrictions were placed on the relationships within or among species. These groups have been resolved as closely related in recent studies and they also correspond to previously recognized subgenera (Rehder, 1940). In the third topology, the 'largeclades' constraint, each of two major lineages resolved in recent studies, one including the two cherry species (*P. avium* and *P. cerasus*) and one including the rest, was constrained as monophyletic, but no other restrictions were imposed. For both *S-RNase* and *SFB* alleles, the shortest unconstrained trees were significantly better (P <0.0001 in all cases) than any tree consistent with any of these constraints, as assessed by the K–H, Templeton, and winning-sites tests.

Variability analyses

When comparing the *S-RNase* and *SFB* genes from *P. tenella* S_8- and *P. avium* S_1-haplotypes, Šurbanovski *et al.* (2007), observed no amino acid differences between the S-RNases but 12 amino acid differences between the SFBs. In order to address the generality of this observation, the per site non-synonymous (K_a) and synonymous (K_s) values for pairs of closely related *S*-haplotypes (those showing a K_s value smaller than 0.1 for either the *S-RNase* or the *SFB*) was determined. There are 20 such *S*-haplotype pairs (Table 4). There is no tendency for an

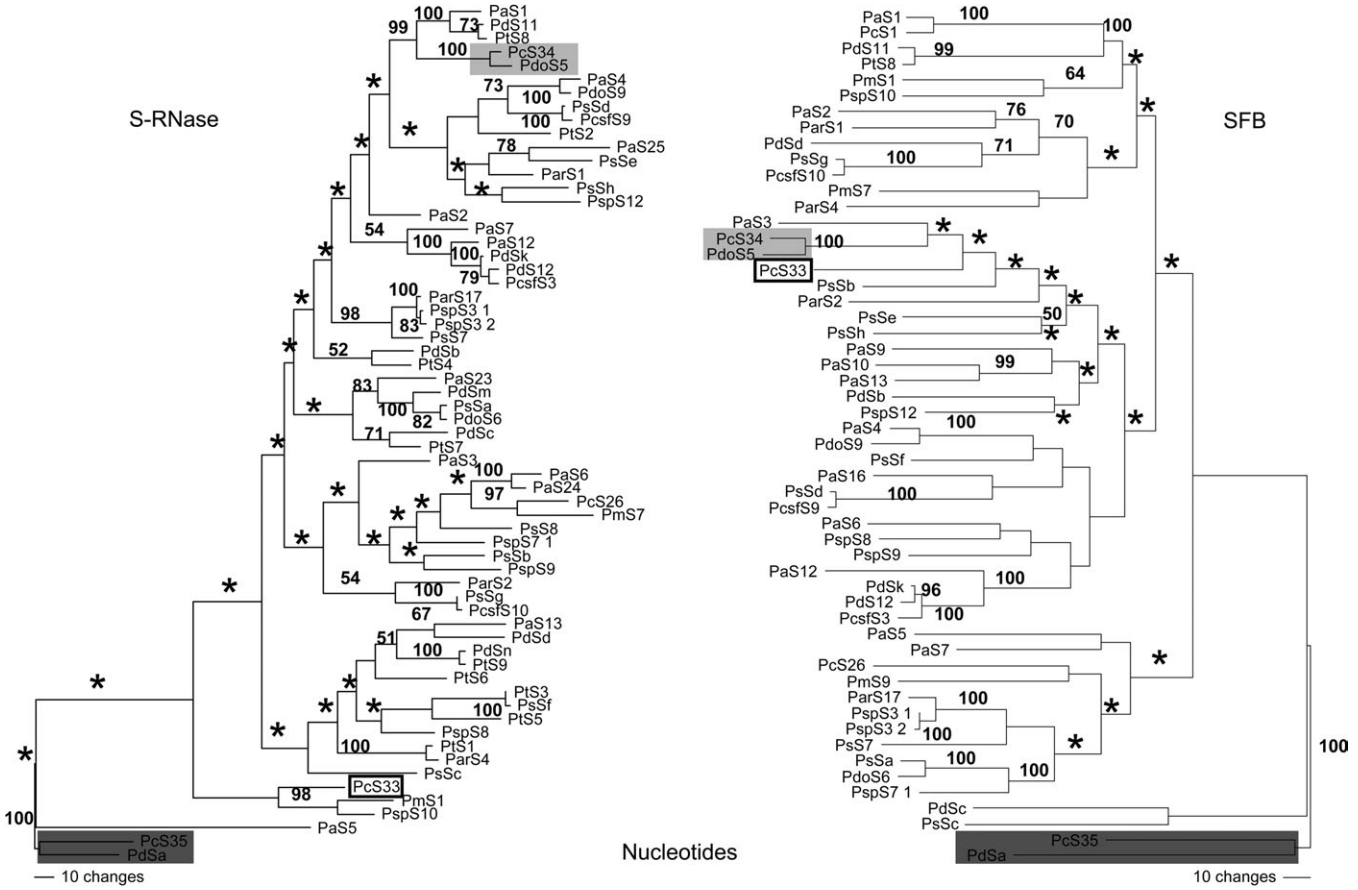

Fig. 7. Phylogenetic trees based on phylogenetic analysis of nucleotide sequences of *S-RNase* (left) and *SFB* (right) alleles for species of *Prunus*. Numbers above, below, or adjacent to branches are bootstrap support values greater than 50%; asterisks indicate branches that collapsed in the strict consensus tree from each analysis. The positions of PcS33, PcS34, and PcS35 are indicated, respectively, by open boxes, lightly shaded boxes, and darkly shaded boxes. Left: Single most parsimonious tree (l=2366, ci excluding autapomorphies=0.3271, ri=0.5263) based on *S-RNase* alleles. The nucleotide sequences for three novel *P. cerasus* (*PcS-RNase*) alleles presented in this study were aligned with 12 *S-RNase* alleles from *P. avium* (*PaS1-RNase*, AB028153; *PaS2-RNase*, AB010304; *PaS3-RNase*, AB010306; *PaS4-RNase*, AB028154; *PaS5-RNase*, AJ298314; *PaS6-RNase*, AB010305; *PaS7-RNase*, EU035974; *PaS12-RNase*, AY259115; *PaS13-RNase*, DQ385842; *PaS23-RNase*, AY259114; *PaS24-RNase*, AY259112; *PaS25-RNase*, AY259113); one from *P. cerasus* (*PcS26-RNase*, EU035975); three from *P. domestica* (*PdoS5-RNase*, AM746946; *PdoS6-RNase*, AM746947; *PdoS9-RNase*, AM746948); nine from *P. dulcis* (*PdSa-RNase*, AB026836; *PdSb-RNase*, AB011469; *PdSc-RNase*, AB011470; *PdSd-RNase*, AB011471; *PdSk-RNase*, AB252409; *PdSm-RNase*, DQ099895; *PdSn-RNase*, DQ093825; *PdS11-RNase*, AM231660; *PdS12-RNase*, AM746949); two from *P. mume* (*PmS1-RNase*, AB101438; *PmS7-RNase*, AB101439); four from *P. armeniaca* (*ParS1-RNase*, AY587561; *ParS2-RNase*, AY587562; *ParS4-RNase*, AY587564; *ParS17-RNase*, EU516388); three from *P. cerasifera* (*PcsfS3-RNase*, AM746943; *PcsfS9-RNase*, AM746944; *PcsfS10-RNase*, AM746945); ten from *P. salacina* (*PsSa-RNase*, AB252411; *PsSb-RNase*, AB252413; *PsSc-RNase*, AB084102; *PsSd-RNase*, AB084103; *PsSe-RNase*, AB280693; *PsSf-RNase*, DQ512911; *PsSg-RNase*, AM746950; *PsSh-RNase*, DQ512914; *PsS7-RNase*, AY781290; *PsS8-RNase*, DQ512913); seven from *P. spinosa* (*PspS8-RNase*, DQ677587; *PspS9-RNase*, DQ677588; *PspS10-RNase*, DQ677589; *PspS12-RNase*, DQ677590; *PspS3-1-RNase*, DQ677584; *PspS3-2-RNase*, DQ677585; *PspS7-1-RNase*, DQ677586); and nine from *P. tenella* (*PtS1-RNase*, DQ983373; *PtS2-RNase*, DQ983374; *PtS3-RNase*, DQ983375; *PtS4-RNase*, DQ983363; *PtS5-RNase*, DQ983364; *PtS6-RNase*, DQ983365; *PtS7-RNase*, DQ983366; *PtS8-RNase*, DQ983367; *PtS9-RNase*, DQ983370). Data set contained 63 taxa and 744 characters, of which 220 were constant, 129 were variable but uninformative, and 395 were parsimony-informative. Right: One of 65 most parsimonious trees (l = 2968, ci excluding autapomorphies = 0.3913, ri = 0.4860) from phylogenetic analysis of nucleotide sequences of *SFB* alleles. The nucleotide sequences for three novel *P. cerasus* (*PcSFB*) alleles presented in this study were aligned with coding sequences for 12 *SFB* alleles from *P. avium* (*PaSFB1*, AY805048; *PaSFB2*, AB111519; *PaSFB3*, AB096857; *PaSFB4*, AB111521; *PaSFB5*, AB111520; *PaSFB6*, AB096858; *PaSFB7*, EU035976; *PaSFB9*, DQ422809; *PaSFB10*, AY805053; *PaSFB12*, AY805054; *PaSFB13*, DQ385844; *PaSFB16*, AY805056); two from *P. cerasus* (*PcSFB1*, DQ827715; *PcSFB26*, EU035977); three from *P. domestica* (*PdoSFB5*, AM746955; *PdoSFB6*, AM746956; *PdoSFB9*, AM746957); seven from *P. dulcis* (*PdSFBa*, AB092966; *PdSFBb*, AB092967; *PdSFBc*, AB079776; *PdSFBd*, AB081648; *PdSFBk*, AB252408; *PdSFB11*, EF061758; *PdSFB12*, AM746959); three from *P. mume* (*PmSFB1*, AB101440; *PmSFB7*, AB101441; *PmSFB9*, AB092645); four from *P. armeniaca* (*ParSFB1*, AY587563; *ParSFB2*, AY587562; *ParSFB4*, AY587565; *ParSFB17*, EU516388); three from *P. cerasifera* (*PcsfSFB3*, AM746952; *PcsfSFB9*, AM746953; *PcsfSFB10*, AM746954); nine from *P. salicina* (*PsSFBa*, AB252410; *PsSFBb*, AB252412; *PsSFBc*, DQ849084; *PsSFBd*, AM746962; *PsSFBe*, AB280794; *PsSFBf*, DQ849089; *PsSFBg*, AM746963; *PsSFBh*, DQ849118; *PsSFB7*, DQ849085); seven from *P. spinosa* (*PspSFB8*, DQ677587; *PspSFB9*, DQ677588; *PspSFB10*, DQ677589; *PspSFB12*, DQ677598; *PspSFB3-1*, DQ677616; *PspSFB3-2*, DQ677615; *PspSFB7-1*, DQ677595), and one from *P. tenella* (*PtSFB8*, DQ983369). Data set contained 54 taxa and 1161 characters, of which 337 were constant, 230 were variable but uninformative, and 594 were parsimony-informative.

Table 4. *Synonymous (K_s) and non-synonymous (K_a)* per site divergence rates for the *S-RNase and SFB genes from closely related S-haplotype pairs*

Prunus sequences		*S-RNase*		*SFB*	
		K_s	K_a	K_s	K_a
P. avium S_1	*P. dulcis* S_{11}	0.0326	0.0000	0.0588	0.0151
P. avium S_1	*P. tenella* S_8	0.0551	0.0000	0.0519	0.0185
P. avium S_4	*P. domestica* S_9	0.1134	0.0060	0.0975	0.0116
P. armeniaca S_{17}	*P. spinosa* $S_{3\text{-}1}$	0.0319	0.0030	0.0396	0.0083
P. armeniaca S_{17}	*P. spinosa* $S_{3\text{-}2}$	0.0319	0.0060	0.0464	0.0100
P. cerasus S_{26}	*P. spinosa* S_9	0.0645	0.1366	0.2622	0.1125
P. cerasus S_{26}	*P. mume*S_7	0.0912	0.1180	0.2194	0.0919
P. cerasus S_{34}	*P. domestica* S_5	0.0979	0.0090	0.0683	0.0166
P. dulcis S_k	*P. dulcis* S_{12}	0.0215	0.0030	0.0000	0.0000
P. dulcis S_k	*P. cerasus* S_3	0.0437	0.0030	0.0192	0.0050
P. dulcis S_{11}	*P. tenella* S_8	0.0437	0.0000	0.0322	0.0033
P. dulcis S_{12}	*P. cerasus* S_3	0.0215	0.0059	0.0192	0.0050
P. mume S_1	*P. spinosa* S_{10}	0.0957	0.0374	0.2688	0.0910
P. salicina S_a	*P. domestica* S_6	0.0324	0.0000	0.0063	0.0134
P. salicina S_d	*P. cerasus* S_9	0.0211	0.0090	0.0064	0.0017
P. salicina S_g	*P. cerasus* S_{10}	0.0215	0.0000	0.0131	0.0033
P. salicina S_7	*P. spinosa* $S_{3\text{-}1}$	0.0883	0.0304	0.1907	0.0689
P. salicina S_7	*P. spinosa* $S_{3\text{-}2}$	0.0883	0.0335	0.1991	0.0707
P. spinosa $S_{3\text{-}1}$	*P. spinosa* $S_{3\text{-}2}$	0.0000	0.0030	0.0065	0.0017
P. spinosa S_9	*P. spinosa* $S_{7\text{-}1}$	0.0992	0.1262	0.2299	0.1060

increased rate of non-synonymous or synonymous mutation at one of the genes compared with the other (Non-parametric Sign test, $P > 0.05$ in both cases).

Since there is no evidence for an increased rate of mutation at the *SFB* gene when compared with the *S-RNase* gene, it is conceivable that a rare recombination event could be responsible for the discrepancy observed by Šurbanovski *et al*. (2007). Using 22 *Prunus* *S*-haplotypes, Nunes *et al*. (2006) noticed that the history of the two genes is positively correlated, although not highly correlated. When using all pair-wise K_s values, the Pearson correlation coefficient was +0.628 (n=231; P <0.01), and when using all pair-wise K_a values, the Pearson correlation coefficient was +0.759 (n=231; P <0.01). This observation is suggestive of recombination at one or both genes. Using the same methodology but a larger data set (n=48), the correlation coefficient was now even lower. When using all pair-wise K_s values, the Pearson correlation coefficient was +0.346 (n=1128; P <0.01) and when using all pair-wise K_a values, the Pearson correlation coefficient was +0.673 (n=1128; P <0.01). Therefore recombination cannot be ruled out as one conceivable reason for the discrepancy observed by Šurbanovski *et al*. (2007). Nevertheless, it should be noted that for both the *S-RNase* and *SFB* gene there is no evidence for the clustering of synonymous or non-synonymous mutations, as expected under the hypothesis of a recent recombination event affecting only a portion of the *SFB* or *S-RNase* gene.

Discussion

Sour cherry is a segmental allotetraploid (Beaver and Iezzoni, 1993) arising from the inter-mating of the diploid sweet cherry and tetraploid ground cherry. Previous studies have shown that six *S*-haplotypes present in sweet cherry (S_1, S_4, S_6, S_9, S_{12}, and S_{13}) are present in sour cherry as well. In this study, it is shown that the sweet cherry S_{14}- and S_{16}-haplotypes are also found in sour cherry, bringing to eight the total number of *S*-haplotypes shared by the two species. The identification in this study of three *S*-haplotypes not previously identified in sour cherry and not found in sweet cherry suggests that these *S*-haplotypes were contributed by the ground cherry (*P. fruticosa*) parent. To address this question, an ongoing survey of the *P. fruticosa* present in the Michigan State University cherry germplasm collection has resulted in the identification of multiple accessions possessing the S_{33}- and S_{35}-haplotypes. However, no individual possessing the S_{34}-haplotype has been identified (T Tsukamoto *et al*., unpublished data).

The finding that the S_{35}-haplotype is functional has significant implications regarding the genetic control of SI and SC in sour cherry. The 'one allele match model' states that for any sour cherry cultivar to be SI it must possess a minimum of three functional *S*-haplotypes (Hauck *et al*., 2006*b*). This is because all pollen carrying at least one matching functional *S*-haplotype is predicted to be incompatible. The landrace cultivars 'Pandy 38', 'Pandy 114' (syn. 'Crisana', 'Köröser'), and 'Tschernokorka' are well documented examples of SI sour cherry cultivars (Redalen, 1984; Lansari and Iezzoni, 1990). Both these cultivars possess three functional *S*-haplotypes, thereby supporting the current hypothesis for the genetic control of SI and SC in sour cherry (Yamane *et al*., 2001; Hauck *et al*., 2006*b*). The three functional *S*-haplotypes in 'Pandy 38' and 'Pandy 114' are S_1, S_4, and S_{35} while the three functional *S*-haplotypes in 'Tschernokorka' are S_9, S_{13}, and S_{35}. This also provides additional evidence that competitive interaction, for example, the compatibility of heteroallelic pollen that is associated with the breakdown of GSI in the Solanaceae, does not occur in tetraploid sour cherry as pollen containing two functional *S*-haplotypes is incompatible. Our result is in contrast to Huang *et al*. (2008) who reported a set of observations compatible with the 'competitive interaction model' in a SC selection of tetraploid *Prunus pseudocerasus*. However, as not all predictions of the model were tested, further data are needed to understand the generality of the 'one allele match model' in *Prunus*.

It is possible that the S_{33}*-RNase* identified in 'Meteor' has the same specificity as the *P. mume* S_{10}*-RNase* (subgenus *Prunus*). Confirmation will require the complete sequence of the *P. mume* S_{10}*-RNase*. However, if these genes do indeed code for identical proteins, this

would represent another example of ancestral specificities shared between *Prunus* subgenera. These findings are consistent with *S*-haplotype divergence predating speciation in *Prunus* (Nunes *et al*., 2006; Vieira *et al*., 2008). *Prunus* species from the same subgenus share, on average, a higher percentage of ancestral specificities than *Prunus* species from different subgenera (Vieira *et al*., 2008). Nevertheless, identical amino acid sequences, as in this case (the comparison of the *S-RNase* from *P. cerasus* S_{33} and *P. mume* S_{10}), were only found between *P. tenella* S_8-RNase and *P. avium* S_1-RNase (Šurbanovski *et al*., 2007), and *P. tenella* S_3-RNase and *P. salicina* S_f-RNase (although the latter two sequences are partial; Vieira *et al*., 2008). The *SFB* of *P. tenella* S_8- and *P. avium* S_1-haplotypes differ, however, by 12 amino acids scattered along the gene (Šurbanovski *et al*., 2007). Such comparison is not possible for *P. cerasus* S_{33}- and *P. mume* S_{10}-*SFB* alleles since the latter sequence is not available. Moreover, the *SFB* sequences of the *P. tenella* S_3- and *P. salicina* S_f-haplotypes are not available.

The inferred rate of synonymous and non-synonymous mutation for closely related haplotypes is not higher at the *SFB* than at the *S-RNase* gene. Therefore, recombination may be the cause of the pattern observed by Šurbanovski *et al*. (2007), although we failed to identify where the putative recombination event occurred.

Phylogenetic analyses provide strong support for the placement of the *P. cerasus* S_{35} and *P. dulcis* S_a-*RNase*s and *SFB*s as a sister group to all the other alleles examined. These specificities are among the oldest ones, and are 15–20 million years old (Vieira *et al*., 2008). The significant conflict between the *S-RNase* and *SFB* data sets, as revealed by the partition homogeneity and tree topology tests, suggest real differences in the underlying phylogenetic histories of the *S-RNase* and *SFB* genes (Fig. 7). These results echo those of Nunes *et al*. (2006). Two observations suggest that homoplasy within one or both data sets, which could result from rapid evolution and/or intragenic recombination involving either or both genes, may be the primary cause of the lack of phylogenetic congruence. First, in phylogenies from both genes, there is generally weak resolution of relationships among alleles, especially for the deeper branches in the trees (Fig. 7). Second, those relationships that are strongly supported tend either to be strongly supported by sequences from both genes (e.g. the sister relationship of *P. cerasus* S_{34} and *P. domestica* S_5) or they are strongly supported by one data set and weakly resolved in the other (e.g. the position of *P. spinosa* $S_{7\text{-}1}$), as reflected by the non-significant results of the topology tests in which each data set was constrained to resolve only groups with 80% or better bootstrap support by the other gene. The fact that the relationships that are strongly supported by either data set, and especially those supported by both data sets, appear to involve recent divergences, while the deeper branches are generally weakly supported, suggests that whatever factors are causing phylogenetic incongruence between the two data sets are also acting to limit our ability to reliably construct more distant relationships among alleles of either gene alone. The lack of congruent topologies of relationship for *S-RNase* and *SFB* alleles does not, in any case, preclude the possibility that the two genes have coevolved to maintain self-incompatibility. Indeed, as expected, since amino acid sites responsible for specificity determination at the *S-RNase* and *SFB* must coevolve, the much higher correlation coefficient for non-synonymous divergence (+0.673) than for synonymous divergence (+0.346) suggests otherwise. As long as the history of amino acid sites important for specificity determination at the *S-RNase* and *SFB* genes is tightly linked, the *S*-haplotype will be functional. For instance, historical intragenic gene conversion may have preferentially occurred in regions where there are no amino acid sites important for specificity determination. It remains, nevertheless, to be determined how much intragenic recombination needs to be argued in order to account for the observed synonymous and non-synonymous correlation coefficients as well as for the overall topology incongruencies. It should be noted that features compatible with recombination have been previously reported at the *S-RNase* and *SFB* genes, although the evidence is still not unequivocal (Vieira *et al*., 2003; Nunes *et al*., 2006; Ortega *et al*., 2006).

The overall amounts of total change and the relative distribution of that change on internal and terminal branches are similar. In both cases, internal branches are generally short compared to many of the terminal branches, suggesting that cladogenic diversification in each gene may have occurred in a common ancestral species, followed by lineage sorting and recombination (not necessarily in that order). In agreement with this view, our phylogenetic analyses of *S-RNase* and *SFB* sequences produced gene trees in which alleles from a single species were not resolved as monophyletic. Such results are expected for genes involved in self-incompatibility among closely related species (Lu, 2001; Vieira *et al*., 2008). Given sufficient divergence time, however, the genes within a particular evolutionary lineage should eventually coalesce. It is known, for example, that within Rosaceae, the *S-RNase* genes of tribe Pyreae (Potter *et al*., 2007) form a clade distinct from those of *Prunus* (Igic and Kohn, 2001). In an attempt to ascertain the phylogenetic magnitude of the lack of correspondence between our gene trees and the presumed phylogeny for these species, a constraint tree approach was used (see Results). Constraining the topologies of the trees in any way to correspond to current understanding of organismal relationships resulted in significantly longer tree lengths. This was true at the level of species, groups of 2–4 closely related species, and two major clades that have been

resolved in recent phylogenetic studies of the genus. Thus, our sampling of five of the roughly 200 species of *Prunus*, which includes representatives of two of the three major lineages within the genus resolved in recent phylogenetic analyses, indicate that neither the *SFB* nor the *S-RNase* locus has attained coalescence at any level below that of the entire genus. Sampling of additional species of *Prunus*, and of species of related genera in Rosaceae, will be required to test this hypothesis and to determine the precise level at which coalescence has occurred.

Supplementary data

The following supplementary data for this article are available at *JXB* online.

Fig. S1. Nucleotide sequence and the deduced amino acid sequence alignments for the *Prunus cerasus* S_{33}-*RNase* and *Prunus mume* S_{10}-*RNase*.

Fig. S2. Nucleotide sequence alignment for the *Prunus cerasus* S_{34}-*RNase*, *P. avium* S_1-*RNase*, *P. dulcis* S_{11}-*RNase*, and *P. tenella* S_8-*RNase*.

Fig. S3. Comparison between downstream sequence of *Prunus cerasus* S_{34}-*RNase* and intergenic sequence between *S-RNase* and *SFB* of *P. avium* S_1, *P. dulcis* S_{11}, and *P. tenella* S_8.

Fig. S4. Nucleotide sequence and the deduced amino acid sequence alignment for the *Prunus cerasus* S_{35}-*RNase* and S_D-*RNase*.

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References

Anderson MA, Cornish EC, Wau S-L, *et al.* 1986. Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiana alata*. *Nature* **321,** 38–44.

Beaver J, Iezzoni AF. 1993. Allozyme inheritance in tetraploid sour cherry (*Prunus cerasus* L.). *Journal of the American Society for Horticultural Science* **118,** 873–877.

Bortiri E, Oh S, Jiang J, Baggett S, Granger A, Weeks C, Buckingham M, Potter D, Parfitt D. 2001. Phylogeny and systematics of *Prunus* (Rosaceae) as determined by sequence analysis of ITS and the chloroplast *trn*L-*trn*F spacer DNA. *Systematic Botany* **26,** 797–807.

Bošković RI, Wolfram B, Tobutt KR, Cerović R, Sonneveld T. 2006. Inheritance and interaction of incompatibility alleles in the tetraploid sour cherry. *Theoretical and Applied Genetics* **112,** 315–326.

de Nettancourt D. 2001. *Incompatibility and incongruity in wild and cultivated plants*. Berlin: Springer.

Entani T, Iwano M, Shiba H, Che F-S, Isogai A, Takayama S. 2003. Comparative analysis of the self-incompatibility (*S*-) locus region of *Prunus mume*: identification of a pollen-expressed F-box gene with allelic diversity. *Genes to Cells* **8,** 203–213.

Hauck NR, Yamane H, Tao R, Iezzoni AF. 2002. Self-compatibility and incompatibility in tetraploid sour cherry (*Prunus cerasus* L.). *Sexual Plant Reproduction* **15,** 39–46.

Hauck NR, Ikeda K, Tao R, Iezzoni AF. 2006*a*. The mutated S_1-haplotype in sour cherry has an altered *S*-haplotype specific F-box protein gene. *Journal of Heredity* **97,** 514–520.

Hauck NR, Yamane H, Tao R, Iezzoni AF. 2006*b*. Accumulation of non-functional *S*-haplotypes results in the breakdown of gametophytic self-incompatibility in tetraploid *Prunus*. *Genetics* **172,** 1191–1198.

Huang S-X, Wu H-Q, Li Y-R, Wu J, Zhang S-J, Heng W, Zhang S-L. 2008. Competitive interaction between two functional S-haplotypes confer self-compatibility on tetraploid Chinese cherry (*Prunus pseudocerasus* Lindl.CV. Nanjing Chuisi). *Plant Cell Reports* **27,** 1075–1085.

Iezzoni AF. 2005. Acquiring cherry germplasm from Central and Eastern Europe. *HortScience* **40,** 304–308.

Igic B, Kohn JR. 2001. Evolutionary relationships among self-incompatibility RNases. *Proceedings of the National Academy of Sciences, USA* **98,** 13167–13171.

Ikeda K, Igic B, Ushijima K, Yamane H, Hauck NR, Nakano R, Sassa H, Iezzoni AF, Kohn JR, Tao R. 2004*a*. Primary structure futures of the *S* haplotype-specific F-box protein, *SFB*, in *Prunus*. *Sexual Plant Reproduction* **16,** 235–243.

Ikeda K, Ushijima K, Yamane H, Tao R, Hauck NR, Sebolt AM, Iezzoni AF. 2005. Linkage and physical distances between the *S*-haplotype *S-RNase* and *SFB* genes in sweet cherry. *Sexual Plant Reproduction* **17,** 289–296.

Ikeda K, Watari A, Ushijima K, Yamane H, Hauck NR, Iezzoni AF, Tao R. 2004*b*. Molecular markers for the self-compatible $S^{4'}$-haplotype, a pollen-part mutant in sweet cherry (*Prunus avium* L.). *Journal of the American Society for Horticultural Science* **129,** 724–728.

Lai Z, Ma W, Han B, Liang L, Zhang Y, Hong G, Xue Y. 2002. An F-box gene linked to the self-incompatibility (*S*) locus of *Antirrhinum* is expressed specifically in pollen and tapetum. *Plant Molecular Biolology* **50,** 29–42.

Lansari A, Iezzoni A. 1990. A preliminary analysis of self-incompatibility in sour cherry. *HortScience* **25,** 1636–1638.

Lu Y. 2001. Roles of lineage sorting and phylogenetic relationship in the genetic diversity at the self-incompatibility locus of Solanaceae. *Heredity* **86,** 195–205.

McClure BA, Haring V, Ebert PR, Anderson MA, Simpson RJ, Sakiyama F, Clarke AE. 1989. Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. *Nature* **342,** 955–957.

McClure BA, Gray JE, Anderson MA, Clarke AE. 1990. Self-incompatibility in *Nicotiana alata* involves degradation of pollen rRNA. *Nature* **347,** 757–760.

Nunes MDS, Santos RAM, Ferreira SM, Vieira J, Vieira C. 2006. Variability patterns and positively selected sites at the gametophytic self-incompatibility pollen *SFB* gene in a wild self-incompatible *Prunus spinosa* (Rosaceae) population. *New Phytologist* **172,** 577–587.

Olden EJ, Nybom N. 1968. On the origin of *Prunus cerasus* L. *Hereditas* **59,** 327–345.

Ortega E, Boskovic RI, Sargent DJ, Tobutt KR. 2006. Analysis of *S-RNase* alleles of almond (*Prunus dulcis*): characterization of new sequences, resolution of synonyms and evidence of intragenic recombination. *Molecular and Genetic Genomics* **276,** 413–426.

Potter D, Eriksson T, Evans RC, *et al.* 2007. Phylogeny and classification of Rosaceae. *Plant Systematics and Evolution* **266,** 5–43.

Redalen G. 1984. Fertility in sour cherries. *Gartenbauwissenschaft* **49,** 212–217.

Richman AD, Uyenoyama MK, Kohn JR. 1996. Allelic diversity and gene genealogy at the self-incompatibility locus in the Solanaceae. *Science* **273,** 1212–1216.

Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R. 2003. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19,** 2496–2497.

Sassa H, Hiranao H, Ikehashi H. 1992. Self-incompatibility related RNases in styles of Japanese pear (*Pyrus serotina* Rehd.). *Plant and Cell Physiology* **33,** 811–814.

Sijacic P, Wang X, Skirpan AL, Wang Y, Dowd PE, McCubbin AG, Huang S, Kao T-h. 2004. Identification of the pollen determinant of S-RNase-mediated self-incompatibility. *Nature* **429,** 302–305.

Sonneveld T, Robbins TP, Boskovic R, Tobutt KR. 2001. Cloning of six cherry self-incompatibility alleles and development of allele specific PCR detection. *Theoretical and Applied Genetics* **102,** 1046–1055.

Sonneveld T, Tobutt KR, Robbins TP. 2003. Allele-specific PCR detection of sweet cherry self-incompatibility (*S*) alleles S_1 to S_{16} using consensus and allele-specific primers. *Theoretical and Applied Genetics* **107,** 1059–1070.

Sutherland BG, Robbins TP, Tobutt KR. 2004. Primers amplifying a range of *Prunus S*-alleles. *Plant Breeding* **123,** 582–584.

Šurbanovski N, Tobutt KR, Konstantinović M, Maksimović V, Sargent DJ, Stevanović V, Ortega I, Bošković RI. 2007. Self-incompatibility of *Prunus tenella* and evidence that reproductively isolated species of *Prunus* have different SFB alleles coupled with an identical *S*-RNase allele. *The Plant Journal* **50,** 723–734.

Swofford DL. 2002. *PAUP*: phylogenetic analysis using parsimony (and other methods),* version 4.0b10. Sunderland: Sinauer.

Tao R, Yamane H, Sugiura A, Murayama H, Sassa H, Mori H. 1999. Molecular typing of *S*-alleles through identification, characterization and cDNA cloning for S-RNases in sweet cherry. *Journal of the American Society for Horticultural Science* **124,** 224–233.

Thompson J, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The ClustalX window interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25,** 4876–4882.

Tobutt KR, Boskovic R, Cerovic R, Sonneveld T, Ruzic D. 2004. Identification of incompatibility alleles in the tetraploid species sour cherry. *Theoretical and Applied Genetics* **108,** 775–785.

Tsukamoto T, Hauck NR, Tao R, Jiang N, Iezzoni AF. 2006. Molecular characterization of three non-functional *S*-haplotypes in sour cherry (*Prunus cerasus*). *Plant Molecular Biology* **62,** 371–383.

Tsukamoto T, Tao R, Iezzoni AF. 2008. PCR markers for mutated *S*-haplotypes enable discrimination between self-incompatible and self-compatible sour cherry selections. *Molecular Breeding* **21,** 67–80.

Ushijima K, Sassa H, Dandekar AM, Gradziel TM, Tao R, Hirano H. 2003. Structural and transcriptional analysis of the self-incompatibility locus of almond: identification of a pollen-expressed F-box gene with haplotype-specific polymorphism. *The Plant Cell* **15,** 771–781.

Ushijima K, Sassa H, Tao R, Yamane H, Dandekar AM, Gradziel TM, Hirano H. 1998. Cloning and characterization of cDNAs encoding *S*-RNases in almond (*Prunus dulcis*): primary structure features and sequence diversity of the *S*-RNases in Rosaceae. *Molecular and General Genetics* **260,** 261–268.

Ushijima K, Sassa H, Kusaba M, Tao R, Gradziel TM, Dandekar AM, Hirano H. 2001. Characterization of the *S*-locus region of almond (*Prunus dulcis*): analysis of a somaclonal mutant and a cosmid contig for an *S*-haplotype. *Genetics* **158,** 379–386.

Vaughan SP, Bošković RI, Gisbert-Climent Russell K, Tobutt KR. 2008. Characterization of novel *S*-alleles from cherry (*Prunus avium* L.). *Tree Genetics and Genomes* **4,** 531–541.

Vieira CP, Charlesworth D, Vieira J. 2003. Evidence for rare recombination at the gametophytic self-incompatibility locus. *Heredity* **91,** 262–267.

Vieira J, Fonseca NA, Santos RAM, Habu T, Tao R, Vieira CP. 2008. The number, age, sharing and relatedness of *S*-locus specificities in *Prunus*. *Genetics Research* **90,** 17–26.

Wheeler D, Newbigin E. 2007. Expression of 10 *S*-class *SLF*-like genes in *Nicotiana alata* pollen and its implications for understanding the pollen factor of the *S* locus. *Genetics* **177,** 2171–2180.

Wünsch A, Hormaza JI. 2004. Cloning and characterization of genomic DNA sequences of four self-incompatibility alleles in sweet cherry (*Prunus avium* L.). *Theoretical and Applied Genetics* **108,** 299–305.

Xia X, Xie Z. 2001. DAMBE: Software package for data analysis in molecular biology and evolution. *Journal of Heredity* **92,** 371–373.

Xue Y, Carpenter R, Dickinson HG, Coen ES. 1996. Origin of allelic diversity in *Antirrhinum S* locus RNases. *The Plant Cell* **8,** 805–814.

Yamane H, Ikeda K, Hauck NR, Iezzoni AF, Tao R. 2003*a*. Self-incompatibility (*S*) locus region of the mutated S^6-haplotypes of sour cherry (*Prunus cerasus*) contains a functional pollen *S*-allele and a non-functional pistil *S*-allele. *Journal of Experimental Bototany* **54,** 2431–2434.

Yamane H, Ikeda K, Ushijima K, Sassa H, Tao R. 2003*b*. A pollen-expressed gene for a novel protein with an F-box motif that is very tightly linked to a gene for S-RNase in two species off cherry, *Prunus cerasus* and *P. avium*. *Plant and Cell Physiology* **44,** 764–769.

Yamane H, Tao R, Sugiura A, Hauck NR, Iezzoni AF. 2001. Identification and characterization of *S*-RNases in tetraploid sour cherry (*Prunus cerasus*). *Journal of the American Society for Horticultural Science* **126,** 661–667.

Yamane H, Ushijima K, Sassa H, Tao R. 2003*c*. The use of *S*-haplotype-specific F-box protein gene, *SFB*, as a molecular marker for *S*-haplotypes and self-compatibility in Japanese apricot (*Prunus mume*). *Theoretical and Applied Genetics* **107,** 1357–1361.